Molecular mechanisms regulating dendrite architecture of mature pyramidal neurons in the mouse hippocampus

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‘Life is all memory,
except for the one present moment
that goes by you so quickly
you hardly catch it going.’
Tennessee Williams

für Thomas Michaelsen

Contents

1 ABSTRACT

Pyramidal neurons are highly complex cells. Their elaborate architecture depends on a tightly regulated balance between stability and plasticity, thereby allowing proper signal transduction and the refinement of neuronal networks due to experience. However, the underlying signaling mechanisms are only partly resolved.

In the current study, I analyzed whether the ratio of the neurotrophin receptors TrkB and $p75^{NTR}$ modulates the morphology of mature pyramidal neurons in the mouse hippocampus. I focused in particular on the truncated kinase-lacking splice variant TrkB.T1. While the overexpression of $p75^{NTR}$ reduced dendritic complexity and spine density, TrkB had the opposite effect. Interestingly, the kinase-lacking receptor T1 induced both positive (spines) and negative (dendrites) morphological alterations. Remarkably, the changes in neuronal morphology were restored by the concomitant expression of T1 and $p75^{NTR}$.

The question, how external signals could be translated into morphological alterations was addressed in the second part of my work. I concentrated on the actin-binding protein profilin. In mammalian brains, two profilin isoforms (PFNI, PFNIIa) are expressed. Especially the role of the brain specific isoform PFNIIa for neuronal morphology is still unresolved. RNAi-mediated knockdown of PFNIIa decreased the number of dendrites and spines. Notably, the concomitant expression of PFNI rescued the loss of spines, but not of dendrites. In order to further specify redundant and discrete functions of PFNI and PFNIIa, I investigated their role in $p75^{NTR}$-mediated structural changes. The results indicate that PFNI and PFNIIa cooperate in preventing distinct aspects of the $p75^{NTR}$-dependent morphological alterations: PFNI in spines and PFNIIa in dendrites.

In summary, the results show that mature neurons use a tightly balanced expression of neurotrophin receptors to control their morphology. Remarkably, changes in dendrites and spines seem to be regulated independently by the use of different actin binding molecules.

Zusammenfassung

Die komplexe Zellgestalt von Neuronen unterliegt einem Gleichgewicht zwischen Stabilität und Veränderung. Dies garantiert eine verlässliche Signaltransduktion und erlaubt zugleich strukturelle Anpassungsfähigkeit, die die erfahrungsabhängige Reorganisation von neuronalen Netzen ermöglicht.

Der Einfluss der Expressionslevel von Neurotrophinrezeptoren ($p75^{NTR}$ und TrkB) auf die Morphologie von Pyramidenneuronen im Hippokampus der Maus war Gegenstand dieser Arbeit. Ein besonderer Fokus lag auf der verkürzten Kinase-defizienten Variante TrkB.T1. Während die Überexpression des $p75^{NTR}$ die Anzahl der Dendriten ebenso wie der dendritischen *spines* verringerte, rief TrkB einen gegenteiligen Effekt hervor. Die Überexpression von T1 jedoch induzierte sowohl negative (Dendriten) als auch positive (*spines*) Veränderungen. Bei einer Coexpression von $p75^{NTR}$ und T1 hingegen blieb die neuronale Morphologie unbeeinflußt.

Im Weiteren wurde in dieser Arbeit untersucht, wie Ligand-Rezeptor Interaktionen in morphologische Veränderungen übersetzt werden können. Die Rolle des Aktin-bindenden Proteins Profilin für die neuronale Morphologie stand hier im Mittelpunkt. Zwei verschiedene Profiline sind im Säugergehirn bekannt (PFNI, PFNIIa). Hierbei ist besonders die Funktion der gehirnspezifischen Form PFNIIa ungeklärt. Eine RNAi-induzierte Hemmung der PFNIIa Genexpression führte zu einem Verlust von Dendriten und *spines*. Einer Verringerung der *spine*-Dichte konnte durch die gleichzeitige Überexpression von PFNI entgegengewirkt werden, nicht jedoch dem Verlust von Dendriten. Eine genauere Untersuchung der spezifischen Funktionen von PFNI und PFNIIa erfolgte am Beispiel $p75^{NTR}$-vermittelter struktureller Veränderungen. Die Überexpression von PFNI konnte hier die Verringerung der *spine*-Dichte verhindern, PFNIIa den Verlust von Dendriten.

Die Ergebnisse dieser Arbeit zeigen, dass die Feinabstimmung der Expression von Neurotrophinrezeptoren eine Modulation der neuronalen Morphologie ermöglicht. Zugleich scheint es, dass verschiedene Aktin-bindende Proteine eine unabhängige Beeinflussung von Dendriten und *spines* erlauben.

2 INTRODUCTION

2.1 The structure of pyramidal neurons

"The goal of neural science is to understand the mind – how we perceive, move, think, and remember"(Kandel et al., 2000). The human brain consists of more than 100 billion individual nerve cells interconnected to systems that control as diverse functions as movement or the formation of memory. Among the most extensively studied nerve cells involved in cognitive processes are pyramidal neurons. These structurally highly complex cells are abundant in fish, birds, reptiles and all mammals, indicating that their core functions have been preserved even as they evolved to perform specialized and diverse tasks (see Box 1). They are found in most mammalian forebrain structures, including the cerebral cortex, the hippocampus and the amygdala, but not the olfactory bulb, the striatum, the midbrain, the hindbrain or the spinal cord. Hence, pyramidal neurons occur primarily in structures associated with advanced cognitive functions.

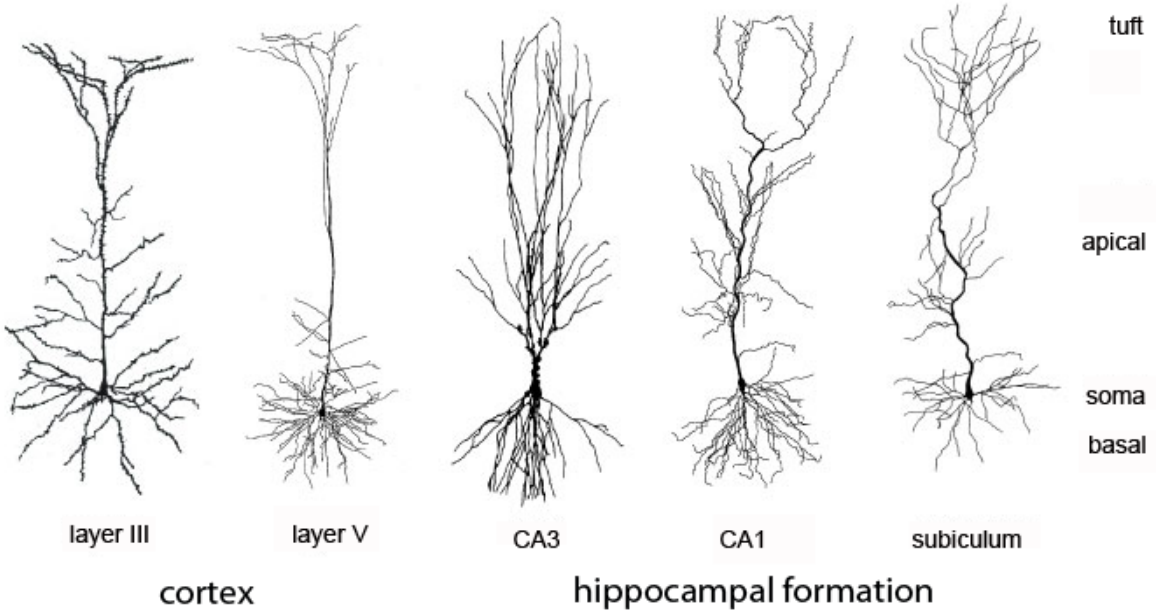


Figure 1 | Pyramidal-neuron structure
Depicted are the structures of pyramidal neurons from different cortical areas. Each pyramidal cell has basal and apical dendrites and an apical tuft, but there are considerable differences between the pyramidal neurons shown. Layer V pyramidal neurons from the cerebral cortex have longer but less branched apical dendrites than layer II/III pyramidal cells. The apical dendrites of pyramidal cells from the CA3 region of the hippocampus branch closer to the soma than those of neurons in the CA1 region. They also show a characteristic cluster of large spines in the first 100 μm of the apical dendrite. All cells displayed are from rat, except the layer III neuron, which is from rabbit. (adapted from Spruston, 2008).

Box | 1 *"The pyramidal cell, or psychic cell, possesses specific characteristics that are never absent …as one ascends the animal scale the psychic cell becomes larger and more complex; it's natural to attribute this progressive morphological complexity, in part at least, to its progressive functional state…it can thus be considered probable that the psychic cell performs its activity more amply and usefully the larger the number of somatic and collateral dendrites that it offers and the more numerous, long and branched the collaterals emitted by its axon" (Ramon y Cajal, 1893)*

Therefore, understanding how these neurons function is necessary to elucidate the neuronal basis of such sophisticated processes (Spruston, 2008). Pyramidal cells are characterized by a pyramidal shaped cell body (soma) and a complex dendritic tree consisting of two distinct domains: the basal and the apical dendrites, originating from the base or the apex of the cell body, respectively (Figure 1). All pyramidal cells have several relatively short basal dendrites, and one apical dendrite giving rise to various oblique branches. The dendrites of pyramidal neurons are studded with thousands of spines, tiny, proto-plasmatic protrusions that receive most of the excitatory synaptic input on these cells. Spines are very heterogeneous in size and shape (see Box 2) and compartmentalize postsynaptic responses in pyramidal neurons. These basic features are maintained between all pyramidal cells. However, they can vary considerably between cortical regions and species (Figure 1). The complexity of the dendritic tree as well as the number of spines are increased in higher cognitive brain areas as the prefrontal cortex. Indeed, the most elaborate and spiny dendrites have been observed in humans (Spruston, 2008). Remarkably, the correlation between the complexity of pyramidal neurons and higher cognitive functions has already been suggested by Santiago Ramón y Cajal more than hundred years ago (see Box 1).

Pyramidal cells receive inhibitory GABA-ergic (γ-aminobutyric acid) input on their soma and axon, whereas excitatory synapses are formed primarily at dendritic spines. Interestingly, proximal dendrites receive excitatory input from the same or adjacent areas, whereas the distal apical tuft receives input from distant cortical or thalamic areas. This indicates that the pyramidal neurons might be designed to respond to coincident input to the tuft and to more proximal domains. In addition, input at the tuft might control responsiveness to more proximal domains (Spruston, 2008).

During the last 30 years, pyramidal neurons of the hippocampus have become the most extensively studied neurons in the brain. A reason for this can be found in the fundamental role of this brain area in memory formation. However, another reason becomes obvious with respect to the special anatomy of this part of the brain (Figure 2). First of all, all principle cells – pyramidal cells in the CA1 and CA3 subfield and granule cells of the dentate gyrus – are organized in a single layer. In addition, these neurons are

connected by a trisynaptic loop, with the main axonal projections running perpendicular to the longitudinal axis of the hippocampus (Figure 2). This simple architecture makes it possible to study hippocampal function *in vitro* by the use of transverse slices, where the main circuitry is preserved (Figure 2).

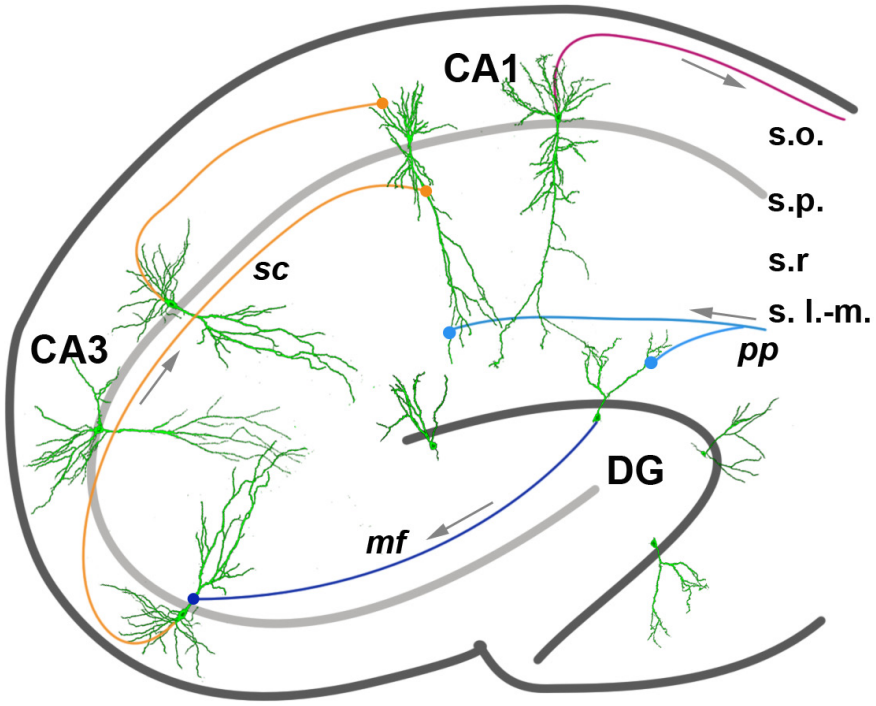


Figure 2 | Schematic illustration of the hippocampal trisynaptic circuit
Granule cells of the dentate gyrus get input from the entorhinal cortex via the perforant path (light blue), and send their axons – mossy fibers (dark blue) – to the pyramidal cells of the CA3 region. CA3 neurons project to the CA1 region via the Schaffer collaterals (orange). In addition, the perforant path projects directly to the apical tufts of CA1 neurons (light blue). CA1 neurons send their axons in turn back to the entorhinal cortex (red).
CA, cornu ammonis; DG, dentate gyrus; mf, mossy fibres; pp, perforant path; sc, schaffer collaterals; s.l.-m., stratum lacunosum-moleculare; s.o., stratum oriens; s.p., stratum pyramidale; s.r., stratum radiatum.

The hippocampus receives its main input to the dentate gyrus from the adjacent entorhinal cortex via the perforant path. The granule cells of the dentate gyrus in turn send their axons (mossy fibers) to pyramidal neurons of the CA3 region. These cells project to pyramidal neurons in the CA1 area via the Schaffer collaterals. Remarkably, the entorhinal cortex is in addition the major output area of the hippocampus. Interestingly, CA1 pyramidal neurons receive input to the apical tuft from the entorhinal cortex, whereas the more proximal dendrites receive input from the CA3 region via the Schaffer collaterals (Figure 2). About 5000 CA3 pyramidal neurons axons converge on a single CA1 cell (Kandel et al., 2000). CA3 neurons more distant from the CA1 region project

primarily to apical dendrites, whereas CA3 cells closer to CA1 project mostly to basal dendrites (Ishizuka et al., 1990;Li et al., 1994) (Figure 2).

Dendrites are the main structures on the nerve cell providing it with synaptic input. Hence, dendritic length and complexity determine the number of synaptic contacts (Hume and Purves, 1981;Purves et al., 1986) and are therefore closely correlated to the proper functioning of neurons. While the dynamics of axon growth and guidance have been studied intensively (Huber et al., 2003), much less is known about how dendrites are growing (for a review see McAllister, 2000). Live-imaging experiments of pyramidal neurons in hippocampal slice cultures showed that dendritic elaboration occurs through highly dynamic structures – so called filopodia – which extend and retract rapidly (Dailey and Smith, 1996). Although many of these filopodia were reabsorbed within minutes, others continued to extend and generated new collateral branches (Dailey and Smith, 1996). These observations suggest that the developing dendritic arbor is highly dynamic, and that some of the lateral dendritic filopodia are precursors of new dendritic branches (Dailey and Smith, 1996). Furthermore, many dendritic filopodia have been shown to make synapses with presynaptic axons (Papa et al., 1995), thereby developing into dendritic spines with characteristic morphologies (for a review see Harris, 1999;Hering and Sheng, 2001) (see Box 2). Spine growth in the adult neocortex has been shown to precede synapse formation *in vivo* (Knott et al., 2006). The final dendritic structure results from a balance between intrinsic developmental programs and local environmental cues modulating the level of activity within neuronal circuits (McAllister, 2000).

Changes in dendritic organization as well as in the number and shape of spines are not restricted to the development of nerve cells but persist beyond adolescence. These changes – known as *plasticity* – are important for the translation of alterations on the level of activity into more persistent changes in neurite structure as required for long-term memory storage (reviewed in Lamprecht and LeDoux, 2004). Activity is known to modulate the formation and maintenance of dendritic branches in the developing and mature brain (Volkmar and Greenough, 1972;Katz et al., 1989;also review in Bailey and Kandel, 1993). Higher-order dendritic branching is indeed significantly increased in cortical pyramidal neurons of rats reared in an enriched environment (Volkmar and Greenough, 1972). Moreover, activity-dependent dendrite formation was observed to be reversible in sympathetic neurons (Vaillant et al., 2002). In young adult mice, substantial rearrangements of dendrites in the superior cervical ganglion were observed over time periods of up to three months, indicating that indeed remodeling takes place well after the

developmental period (Purves et al., 1986). Remarkably, interrupting or modulating synaptic input to distinct sets of dendrites has been shown to regulate their dendritic structure on a very short timescale, as observed in the chick nucleus laminaris (Sorensen and Rubel, 2006).

Like dendrites, dendritic spines were long since considered to be stable structures. However, recent imaging studies revealed an activity-induced growth of filopodia-like structures in CA1 pyramidal neurons (Maletic-Savatic et al., 1999;Engert and Bonhoeffer, 1999). Moreover, spines in mature hippocampal neurons were found to undergo rapid actin-dependent changes in shape (Fischer et al., 1998). Long-term enhancement of synaptic efficacy in the hippocampus has been shown to be a suitable model for studying cellular processes of neuronal plasticity. The induction of long-term potentiation (LTP) via high frequency stimulation of the Schaffer collaterals (Figure 2) leads to an increase in the strength of CA1 synapses which is accompanied by the growth of new spines (Engert and Bonhoeffer, 1999). In an opposite approach, the weakening of synapses (long-term depression, LTD) following a low frequency stimulation protocol in this region leads to the disappearance or shrinkage of existing spines (Nagerl et al., 2004;Zhou et al., 2004).

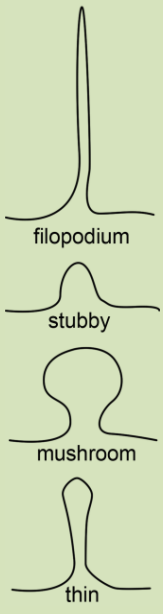
In summary, these observations show that dendritic structure can be modulated in a highly dynamic fashion not only during development but as well in the adult brain. The changes in neuronal structure have been correlated with changes in neuronal activity.

Box | 2 Spine morphology

Spines are tiny, protoplasmatic protrusions receiving over 90% of all excitatory synaptic inputs in the neocortex. The prototypical spine consists of a bulbous head connected to the dendritic shaft by a narrow neck (mushroom spine, see right). Stubby spines without a neck and thin spines lacking a head can be found side by side with mushroom spines along the dendrites (right). Filopodia are believed to be the precursors of spines.

A spine can be seen as a microcompartment for the segregation of postsynaptic responses – as elevated levels of Ca^{2+} – from the apparent dendritic shaft. The geometry of the spine neck might therefore control the kinetics and magnitude of postsynaptic calcium responses.

In general, larger spines have been found to carry larger synapses and to contain a greater diversity of organelles. The postsynaptic density (PSD) occupies ~10% of the spine surface area. Remarkably, spine size seems to be correlated with the size of the PSD and with the number of postsynaptic receptors. Interestingly, LTP induction at single spines by glutamate uncaging produced a long-lasting enlargement of the spine size which might be correlated to the enhancement in synaptic efficacy (for reviews see Hering & Sheng 2003; Cingolani & Goda 2008)



Among the molecular cues especially important for neuronal survival and differentiation, neurotrophins play one of the most prominent roles. They became even more interesting as more and more evidence points to a crucial role also in activity-dependent forms of synaptic as well as structural plasticity.

2.2 Neurotrophins and their receptors

Neurotrophins are involved in the regulation of development, maintenance, and function of the vertebrate nervous system (for a review see Huang and Reichardt, 2001). The discovery of NGF as the first neurotrophic factor (reviewed in Levi-Montalcini, 1987) represented a hallmark in understanding molecular guidance cues and revealed the importance of cellular interactions during development. Initially described as survival factors secreted by the target tissues (Purves et al., 1988), increasing evidence suggests that neurotrophins are as well involved in mechanisms of functional and structural plasticity (for reviews see McAllister et al., 1999;Huang and Reichardt, 2001;Chao, 2003;Lu et al., 2005). In mammals, four different neurotrophins have been described (Figure 3): nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 and 4 (NT-3, NT-4). While all of them bind with equimolar affinity to the pan neurotrophin receptor $p75^{NTR}$ (Rodriguez-Tebar et al., 1991), only one of them interacts preferentially with one of the so called Trk receptors (tropomyosin-related kinase receptors) (reviewed in Bothwell, 1995). Synthesized as precursors, neurotrophins are proteolytically processed to form mature proteins (Seidah et al., 1996a;Seidah et al., 1996b). Remarkably, pro-neurotrophins have been shown to bind with high affinity to the $p75^{NTR}$ thereby inducing apoptosis, whereas the mature proteins preferentially activate Trk receptors promoting cell survival (Lee et al., 2001). Thus, the action of neurotrophins might not only be determined by the expression levels of distinct receptor types (see below) but moreover by proteolytic processing of the proteins themselves. However, it is under a current debate, if pro-neurotrophins are released under normal physiological conditions (Matsumoto et al., 2008;Yang et al., 2009).

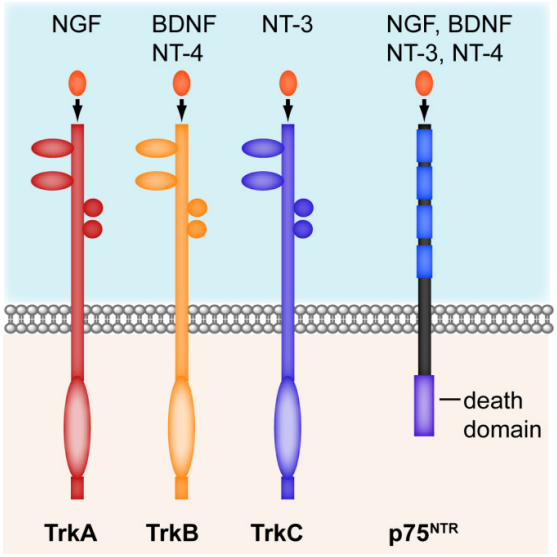


Figure 3 | Neurotrophins and their receptors
Neurotrophins bind selectively to one Tropomyosin-related kinase receptor (Trk), whereas all of them bind to the pan neurotrophin receptor $p75^{NTR}$ with equimolar affinity (Adapted from Chao, 2003).
NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; NT, neurotrophin

2.2.1 Trk-receptors as positive modulators of neuronal structure and function

Neurotrophins have been shown to bind and dimerize Trk receptor tyrosine kinases, resulting in the activation of the intracellular kinase through transphosphorylation. While NGF activates TrkA, BDNF and NT-4 are specific for TrkB. NT-3 preferentially interacts with TrkC but to a lower extend is also able to activate all other neurotrophin receptors. The direct interaction with a dimer of neurotrophins is mediated by the membrane-proximal of two immunoglobulin-like domains (Ultsch et al., 1999;Wiesmann et al., 1999). Endocytosis and transfer of Trk receptors to different membrane compartments control Trk-mediated signaling, especially as many of the important adaptor proteins are localized within distinct membrane compartments (York et al., 2000). Splicing results in additional Trk isoforms. The insertion of a short amino acid sequence in the juxtamembrane region affects ligand specificity by enhancing the binding of non-preferred ligands (Clary and Reichardt, 1994;Strohmaier et al., 1996). Moreover, alternative splicing results in kinase-lacking isoforms of TrkB and TrkC (Klein et al., 1990;Tsoulfas et al., 1993) which will be discussed in detail below.

Trk receptors carry ten conserved tyrosine residues, three of which are involved in controlling the kinase activity of the receptor complex. Phosphorylation of the other residues regulates the interaction with proteins carrying phosphotyrosine-binding (PTB) or Src-homology 2 (SH2) domains (reviewed by Reichardt, 2006). Neurotrophin binding to Trk receptors activates essential intracellular pathways important for neuronal survival and differentiation (Figure 4): Ras, PI3K (phosphatidylinositol 3-kinase), PLC-γ and their downstream effectors are involved in Trk-mediated signaling (reviewed by Huang and Reichardt, 2003). However, ample evidence indicates that they are involved as well in the development and function of synapses. Neurotrophins have been shown to enhance synaptic transmission in the peripheral as well as in the central nervous system (Lessmann et al., 1994;reviewed in Lu, 2003). In particular the role of BDNF in modulating the long-term enhancement of synaptic efficacy in hippocampal pyramidal neurons has been studied intensively. Specifically, BDNF deficient mice show an impairment in hippocampal LTP (Korte et al., 1995a;for reviews see Poo, 2001;Lu, 2003) that could be rescued by reintroduction of exogenous BDNF (Korte et al., 1996). In the visual cortex BDNF has been shown to facilitate LTP (Huber et al., 1998) and attenuated LTD in layer II/III pyramidal neurons of young adult rats (Akaneya et al., 1996;Kumura et al., 2000). At the same time neuronal activity increases the number of TrkB receptors at

the surface of hippocampal neurons thereby promoting the action of BDNF (Du et al., 2000). Interestingly, the activation of TrkB can be seen as a link between changes in synaptic strength and structural alterations. Neurotrophin have in fact been shown to regulate cortical growth in an activity-dependent manner (McAllister et al., 1996). The expression of BDNF in cortical pyramidal neurons induces the sprouting of multiple highly unstable dendrites (Horch et al., 1999;Horch and Katz, 2002). Moreover, different neurotrophins might be involved in regulating distinct aspects of neuronal growth, as BDNF and NT-3 were found to oppose one another in regulating the dendritic growth of pyramidal neurons (McAllister et al., 1997). Deletion experiments targeting TrkB in cortical pyramidal neurons reported dendrite retraction and neuronal loss further underlining its role as a positive modulator of dendrite structure (Xu et al., 2000). Furthermore, the BDNF-TrkB signaling is reported to positively modulate axonal branching (Cohen-Cory and Fraser, 1995;Gallo and Letourneau, 1998) as well as spine density (Tyler and Pozzo-Miller, 2001;Tyler and Pozzo-Miller, 2003).

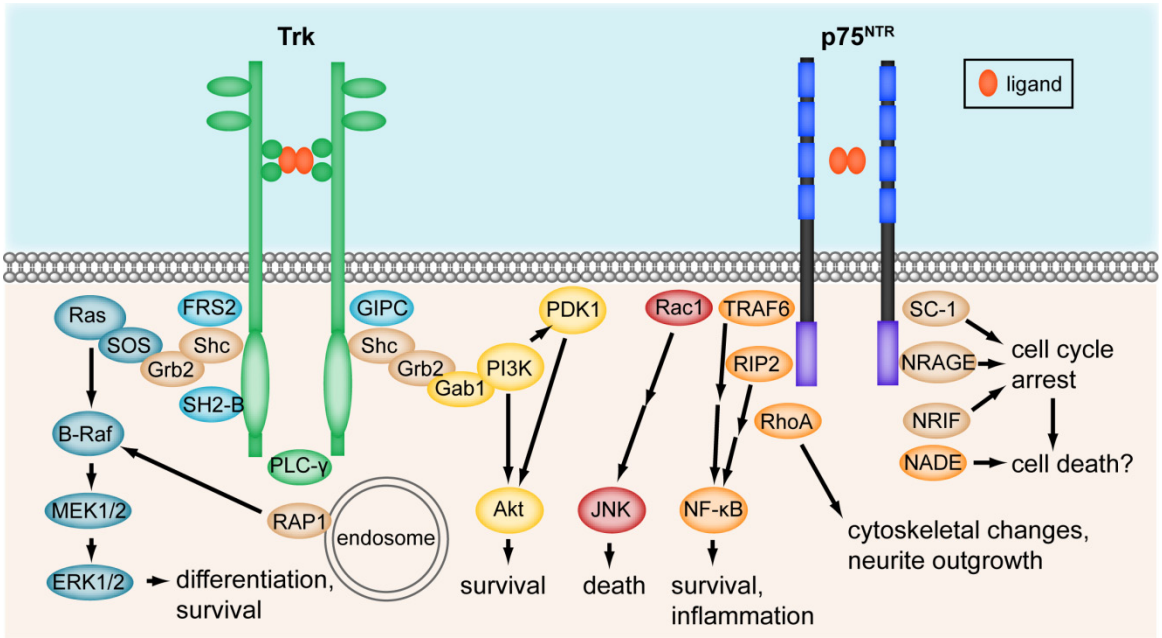


Figure 4 | Neurotrophin receptor signaling
Trk receptors are mediating differentiation and survival through the extracellular signal-regulated kinase (ERK), phospatidylinotitol 3 kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways. The $p75^{NTR}$ actives NF-κB and the Jun N-terminal kinase (JNK). Moreover $p75^{NTR}$ is known to regulate neurite outgrowth through modulation of RhoA activity via the interaction with Rho-GDI1 (Adapted from Chao, 2003).
Akt, protein kinase B; FRS2, fibroblast growth factor receptor substrate 2; Gab1, Grb2-associated binder 1; Grb2, growth factor receptor-bound protein 2; GIPC, GAIP interacting protein, C terminus; MEK, mitogen-activated protein kinase (MAPK)/ERK kinase; NADE, neurotrophin-associated cell death executor; NRIF, neurotrophin-receptor interacting factor; NRAGE, neurotrophin-receptor interacting MAGE homologue; PDK1, phosphoinositide-dependent kinase 1; RIP2, receptor-interacting protein 2; SC-1, Schwann cell protein 1, SH2B, Src-homology 2-B; SOS, son of sevenless; Shc, Src homologous and collagen-like adaptor protein; TRAF-6, tumor necroses factor receptor-associated factor 6.

2.2.2 P75^{NTR}: one neurotrophin receptor but many faces

While the Trk receptors exert well-defined trophic functions, p75^{NTR} is reported to mediate such diverse effects as cell survival and apoptosis (Figure 4). Initially identified as a low-affinity receptor for NGF, p75^{NTR} was later shown to bind all neurotrophins with similar affinity (Rodriguez-Tebar et al., 1990). P75^{NTR} is a member of the tumor necrosis factor family with an extracellular domain comprised of four cystein-rich repeats and a cytoplasmatic tail including a 'death' domain (Figure 3) comparable to those present in other members of this family (He and Garcia, 2004). Interestingly, it has been shown that binding of NGF to p75^{NTR} results in an asymmetric receptor-ligand-complex through the induction of conformational changes that prevent an interaction with a second p75^{NTR} molecule (He and Garcia, 2004).

One of the best characterized functions of p75^{NTR} is the induction of cell death: both in the period of development when programmed cells death contributes to the refinement of neuronal networks (Majdan and Miller, 1999) and during inflammation, seizures or spinal cord injury (Dowling et al., 1999;Roux et al., 1999;Beattie et al., 2002). The latter case was reported to involve the activation of Rac and JNK (Jun N-terminal kinase) (Harrington et al., 2002). Interestingly, pro-neurotrophins have been shown to bind preferentially to p75^{NTR} and to be more effective in inducing p75^{NTR}-dependent apoptosis (Lee et al., 2001;Beattie et al., 2002). In addition, it has been shown that p75^{NTR} is able to promote cell survival in Schwann cells (Khursigara et al., 2001). The trophic as well as apoptotic functions of p75^{NTR} are consistent with the actions of its various coreceptors. The induction of apoptosis by pro-neurotrophins has been shown to involve a ternary complex comprised of pro-neurotrophins, p75^{NTR} and sortilin, an additional receptor for pro-neurotrophins (Nykjaer et al., 2004;Teng et al., 2005). It was reported recently that p75^{NTR} and the Nogo-receptor form a complex mediating the repulsive signaling of myelin based growth inhibitors (MBGI) (Wong et al., 2002) most likely due to the activation of RhoA via p75^{NTR} (Yamashita and Tohyama, 2003). Remarkably, p75^{NTR} was also reported to be a coreceptor for the Trk receptors. Coexpression of p75^{NTR} can increase the affinity of Trk receptors for their neurotrophins and is able to further enhance their ligand specificity (Hempstead et al., 1991;Benedetti et al., 1993;Bibel et al., 1999). The presence of p75^{NTR} potentiates the activation of TrkA by low concentrations of NGF (Davies et al., 1993;Mahadeo et al., 1994). Trk receptors and p75^{NTR} are thought to form a 'high-affinity' binding site by binding simultaneously to one homodimer of

neurotrophins thereby enhancing Trk signaling (Hempstead et al., 1991). However, Wehrman and Colleagues (2007) recently provided new evidence suggesting that the formation of this complex could be sterically impossible.

In addition, $p75^{NTR}$ and Trk receptors have been found to elicit strongly opposing biological responses in neurons (reviewed in Lu et al., 2005) (Figure 5). Numerous reports show Trk receptors as mediators of positive structural and functional plasticity in the developing and adult nervous system (see above). Yet, growing evidence indicates that $p75^{NTR}$ could act as the opposing player of Trk receptors involved in long-term decrease of synaptic efficacy as well as in negatively regulating dendrite structure. This idea is supported by studies in $P75^{NTR}$ knockout mice showing an impairment in the maintenance of long-term depression, (Rosch et al., 2005;Woo et al., 2005). LTP, however, was found to be unaltered in these animals. Furthermore, dendritic complexity and spine density are increased in organotypic hippocampal slice cultures of $p75^{NTR}$ knockout mice (Zagrebelsky et al., 2005). In the same study, the overexpression of $p75^{NTR}$ in pyramidal neurons led to a reduction in dendrite structure and spine number. The underlying signaling pathways that could mediate these $p75^{NTR}$-dependent structural alterations are only in parts resolved. Yet, $p75^{NTR}$ has been shown to modulate the activity of the small GTPase RhoA, thereby providing a possible link to the actin cytoskeleton (Yamashita et al., 1999;Yamashita and Tohyama, 2003;Gehler et al., 2004). In the absence of a ligand, $p75^{NTR}$ was reported to activate RhoA, whereas neurotrophin binding in turn abolished RhoA activity (Yamashita et al., 1999).

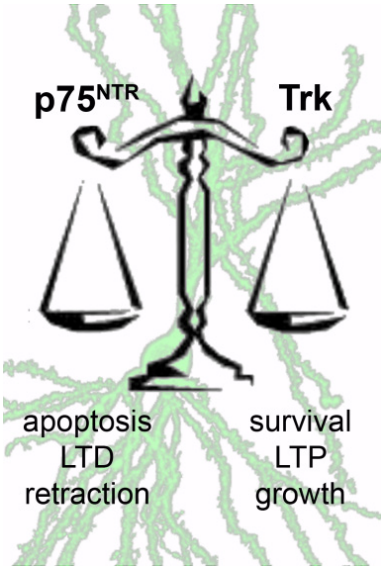


Figure 5 | The antagonistic dual receptor system of $p75^{NTR}$ and Trk receptors
Neurotrophins bind to two distinct types of receptors with often opposing biological responses on the function and structure of neurons.
LTP, long-term potentiation; LTD, long- term depression

Taken together the Trk receptors and the $p75^{NTR}$ emerge as a dual receptor system whose precisely regulated action and expression patterns may provide the neurons with the ability to tightly control both their function and structure (reviewed in Lu et al., 2005;Blochl and Blochl, 2007).

2.2.3 Truncated Trks: the good, the bad and T1

Since the discovery that alternative splicing generates truncated Trk receptors, there is an ongoing debate about their physiological function *in vivo*. Truncated, kinase lacking forms of both TrkB and TrkC are known (TrkB.T1, TrkB.T2 and TrkC.T1) (Klein et al., 1990;Middlemas et al., 1991;Tsoulfas et al., 1993). Interestingly, the expression patterns of full-length and truncated receptors vary considerably during development. While the full-length TrkB (TK+) receptor is the predominant isoform during early development, the expression level of TrkB.T1 (T1) is dynamically upregulated postnatally and constitutes the predominant isoform in the adult brain (Allendoerfer et al., 1994;Escandon et al., 1994;Fryer et al., 1996). However, the precise ratios of TK+ and T1 vary between different brain areas, with septum and hippocampus showing the highest ratio between T1 and TK+ (Fryer et al., 1996).

Initially the role for the truncated receptors has been described as factors restricting the action of the full-length receptors through the formation of heterodimers. Indeed, overexpression studies using T1 suggest a role of the truncated receptor as a dominant negative inhibitor of TK+ signaling (Eide et al., 1996;Drake et al., 1999;Ohira et al., 2001;Haapasalo et al., 2001;Haapasalo et al., 2002;Lahteinen et al., 2002). In addition, T1 has been shown to act as a BDNF scavenging receptor (Klein et al., 1990;Middlemas et al., 1991;Biffo et al., 1995;Eide et al., 1996;Saarelainen et al., 2000a) thereby tightly regulating the action of TK+. Deletion of T1 partially rescues BDNF haploinsufficiency indicating that T1 in fact limits BDNF signaling *in vivo* (Carim-Todd et al., 2009).

The surprising observation that T1 is capable of signaling independently (Baxter et al., 1997;Rose et al., 2003;Ohira et al., 2005;Cheng et al., 2007) was unexpected taking into account that the intracellular domain of this receptor isoform comprises only 23 amino acids. Yet, the amino acid sequence displays 100% sequence conservation between humans, mice, rats and felines (Klein et al., 1990;Middlemas et al., 1991;Baxter et al., 1997). Microphysiometric assays show that both T1 and T2 are capable of ligand-mediated changes in cell physiology (Baxter et al., 1997). These results suggest that the truncated receptors can induce ligand-mediated signal transduction. Moreover, mutational analysis demonstrate that the isoform specific intracellular domains of T1 and T2 are essential for their signaling capability (Baxter et al., 1997). Further evidence for T1 signaling is provided by studies in glia cells. Rose and colleagues (2003) could show a BDNF induced increase in intracellular Calcium levels in astrocytes, which express T1 as

the predominant isoform. These calcium transients were found to be insensitive to blocking of the kinase activity of the full-length TrkB receptor, indicating that they are indeed mediated by T1 (Rose et al., 2003). The authors of this study suggest that an unknown G protein might be involved in the intracellular signaling cascades mediated by T1 upon binding of BDNF. In addition, two different binding partners of T1 were identified, one of them a novel protein named after its interaction with T1 (TIPP, truncated Trk interacting protein) (Kryl and Barker, 2000). Remarkably, affinity purification revealed Rho GDP dissociation inhibitor 1 (Rho GDI1) as a protein directly associated with T1 (Ohira et al., 2005). Upon BDNF binding Rho GDI1 dissociates form the C-terminal tail of T1 thereby controlling the activities of Rho GTPases (Ohira et al., 2005). In astrocytic cultures and glioma cells, T1 indeed has been shown to induce morphological changes in a BDNF-dependent manner by Rho GDI1 (Ohira et al., 2005;Ohira et al., 2006;Ohira et al., 2007).

Interestingly, T1 has as well been shown to affect the morphology of pyramidal neurons (Yacoubian and Lo, 2000;Hartmann et al., 2004). The overexpression of T1 in hippocampal neurons induces the formation of dendritic filopodia (Hartmann et al., 2004). Remarkably, this outgrowth of protrusion seems to involve the action of $p75^{NTR}$, as the expression of a $p75^{NTR}$ mutant lacking the intracellular domain abolished the T1-mediated growth of filopodia (Hartmann et al., 2004). Overexpression studies in ferret cortical slices show that the ratio of T1 to full-length TK+ can act as a switch between two different modes of dendritic growth (Yacoubian and Lo, 2000). Specifically, while the overexpression of TK+ induces the formation of new dendrites in proximal regions of the neurons, T1 overexpression promotes the elongation of already existing dendrites at distal parts of the dendritic tree.

The broad action range of neurotrophins affecting neuronal survival, function and structure is mediated by two different types of neurotrophin receptors – Trk receptors and $p75^{NTR}$. Interestingly, the truncated receptor T1 could add a third regulatory component to this system. T1 might act together with these receptors, to further define their action, probably by heterodimerization or ligand sequestration. Alternatively, T1 could act independently of the other neurotrophin receptors by active signaling itself.

2.3 **The neuronal actin cytoskeleton**

Receptor-ligand interaction can initiate activity-dependent changes in the function and in the structure of pyramidal neurons. However, in order to allow precisely regulated structural changes, the neuronal cytoskeleton needs to be involved. In neurons, the cytoskeleton comprises three distinct but interacting structural components: microtubules, neurofilaments and microfilaments. It is the spatial and temporal modulation of these components enabling nerve cells to maintain as well as to rapidly change their cell architecture.

The actin cytoskeleton exhibits the most diverse composition and organization. While actin microfilaments are found in the entire neuron, they are enriched in cortical regions near the plasma membrane and are particularly concentrated in presynaptic terminals, growth cones and dendritic spines. High concentrations of actin at the membrane are often associated with changes in cell shape (Cooper, 1991), and indeed time-lapse imaging revealed spontaneous as well as activity-dependent structural changes of dendritic filopodia and spines that are depending on actin polymerization (Dailey and Smith, 1996;Fischer et al., 1998;Lendvai et al., 2000;Honkura et al., 2008) (for reviews see Hering and Sheng, 2001;Bonhoeffer and Yuste, 2002). In addition, stimulation protocols leading to the long-lasting enhancement of synaptic efficacy (LTP) have been shown to depend on a dynamic actin cytoskeleton (Kim and Lisman, 1999;Krucker et al., 2000).

Actin in spines is surprisingly dynamic, with 85% being exchanged within 2 min (Halpain et al., 1998;Star et al., 2002). Long-term plastic changes in synaptic efficacy have been shown to induce rapid and persistent reorganization of the spine actin cytoskeleton. Upon the induction of LTP, the ratio of F- and G-actin is shifted towards F-actin accompanied by an increase in spine volume (Fukazawa et al., 2003;Lin et al., 2005). In contrast to this, LTD induction shifts the ratio towards G-actin and results in the shrinkage of spines (Okamoto et al., 2004). CaMKII is one of the molecules that could link functional plasticity to structural changes in the spine cytoskeleton. Activated CaMKII translocates into the spine and is responsible for the activation of multiple signaling pathways involved in LTP maintenance (Silva et al., 1992;Shen and Meyer, 1999;Thalhammer et al., 2006). Remarkably, CaMKII has been shown in addition to function as a structural component that is able to bind and bundle F-actin (Okamoto et al., 2007).

Intriguingly, changes in spine shape most likely decline during postnatal development (Dunaevsky et al., 1999;Lendvai et al., 2000) correlating with a high concentration of F-actin in dendritic spines in the adult brain (Cohen et al., 1985). This implies that the actin cytoskeleton in mature neurons exhibits a stabilizing function that nevertheless retains the potential for morphological plasticity (reviewed in Matus, 2000;Cingolani and Goda, 2008).

2.3.1 Profilins, important modulators of actin filament dynamics

From the very beginning of neuritogenesis to the subtle modifications in the shape of mature spines, a multitude of molecules is involved in regulating microfilament growth or collapse (reviewed in Dillon and Goda, 2005). Among these molecules regulating actin dynamics profilin plays a key role by enhancing nucleotide-exchange of G-actin and providing it to the growing actin filament (Carlsson et al., 1977;Kang et al., 1999). Different profilin genes are expressed in phylogenetically disparate organisms as yeast (Haarer et al., 1990), plants (Staiger et al., 1993) or vertebrates. Specifically, in mammals profilinI (PFNI) expression has been shown to be ubiquitous and essential (Witke et al., 1998;Witke et al., 2001), while in contrast profilinIIa (PFNIIa) shows its highest expression level in the brain. Other tissue specific isoforms can be found in kidney (Di Nardo et al., 2000) or testis (Hu et al., 2001;Obermann et al., 2005). Besides binding actin itself profilins are characterized by their interaction with actin related proteins (ARPs) and two other types of protein binding domains: poly-L-proline (PLP) stretches in proteins of e.g. the Ena/VASP-family, WAVE or the formins, and membrane bound phospho-lipids like phosphatidylinositol-4,5-bisphosphate (PIP2) (Lassing and Lindberg, 1985;reviewed in Jockusch et al., 2007).

Although a variety of interaction partners are known, the specific role of the different profilin isoforms and especially of the brain specific PFNIIa remains poorly understood. Recent evidence has been provided for both pre- and postsynaptic functions of profilins. They have been shown to affect dendritic spine stability *in vitro* and *in vivo*: Experiments in cultured hippocampal neurons showed activity mediated targeting of PFNI (Neuhoff et al., 2005) as well as PFNIIa into spines of excitatory neurons (Ackermann and Matus, 2003). Furthermore, Lamprecht and colleagues (2006) could show a stimulus dependent accumulation of profilin (without further isoforms specification) in spines of neurons in the rat amygdala after fear conditioning. In addition, PFNIIa has been shown to indirectly

interact with the small GTPase RhoA through the RhoA specific kinase ROCK to affect spine morphology in an activity-dependent manner involving NMDA receptor activation (Schubert et al., 2006). On the other hand, Witke and colleagues recently showed in a gene-targeted approach that PFNIIa acts presynaptically by controlling vesicle exocytosis and presynaptic excitability leading to increased novelty-seeking behaviour in PFNIIa knock-out mice (Pilo-Boyl P. et al., 2007).

2.4 Aim of this study

Pyramidal neurons are highly complex cells. Changes in dendritic organization as well as in the number and shape of spines are not restricted to the development of these cells but persist beyond adolescence. These changes – known as *plasticity* – are important for the translation of alterations on the level of activity into more persistent changes in neurite structure as required for long-term memory storage. The work presented in this thesis is focused on two different sets of molecules known to be important for neuronal morphology and is aimed to investigate their importance especially for the structure of mature CA1 pyramidal in organotypic hippocampal slice cultures two weeks in culture.

1) Neurotrophins and their receptors are involved in a variety of cellular functions ranging from differentiation to synaptogenesis and activity-dependent forms of synaptic plasticity. In the current study, I was interested whether the balance in expression levels and activation of different neurotrophin receptors could modulate the morphology of mature pyramidal neurons. In particular the function of the truncated splice variant TrkB.T1, which lacks intracellular kinase activity, should be investigated.

2) The actin cytoskeleton of neurons possesses the capacity to stabilize as well as to allow the dynamic reorganization of neuronal structure described above. Among the actin binding molecules known to modulate actin dynamics, profilin plays a key role. However, the existence of two different profilin isoforms in the mammalian brain long since raised the question, why two profilins are needed. The aim of the current study was to investigate the physiological role of the brain specific isoform PFNIIa in regulating dendrite morphology and spine stability of mature pyramidal neurons. To this end, a loss of function approach inducing RNAi-mediated knockdown of PFNIIa was used. Finally, this study was aimed at combining the insights of both experimental approaches to discover details about how neurotrophin receptors could modulate neuronal structure by regulating actin dynamics.

3 MATERIAL AND METHODS

3.1 Reagents

Agar-Agar	Roth
Ampicillin	MP Biomedical
B27 supplement	Gibco
BME Medium	Gibco
Borax	Sigma
Boric acid	Merck
BSA	Roth
Cytosin-D-Arabinofuranosid hydrochloride	Sigma
Equine donor serum	HyClone (Perbio)
Fetal calf serum (FCS)	PAA Laboratories
5-Fluoro-2'-Deoxyuridine	Sigma
GlutaMAX	Gibco
Goat Serum	Invitrogen
Hank's Balanced salt solution	Gibco
Kanamycin-sulfate	MP Biomedicals
Kynurenic acid	Sigma
Lipofectamine 2000®	Invitrogen
N-methyl-D-aspartic acid (NMDA)	Sigma
Paraformaldehyde	AppliChem
Plasmid preparation kit	Qiagen
Poly-L-lysine	Sigma
Polyvinylpyrrolidone (PVP)	Bio-Rad
Spermidine	Sigma
Triton X-100	Sigma
Trypsin-EDTA 1x	Sigma
Tryptone	MP Biomedicals
Uridine	Sigma
Yeast extract	MP Biomedicals

3.2 Solutions and Media

Organotypic cultures:

Gey's Balanced Salt Solution pH 7.4 (GBSS)

COMPONENT	MOLARITY (mM)
$CaCl_2$ * 2 H_2O	1.5
KCl	5
KH_2PO_4	0.22
$MgCl_2$ * 6 H_2O	1
$MgSO_4$ * 7 H_2O	0.28
NaCl	137
Na_2HCO_3	2.7
Na_2HPO_4	0.86
D-Glucose	5.5

Kynurenic acid

Dissolve 946 mg Kynurenic acid in 5 ml 1 M NaOH, stir 2-3 h, add 45 ml $H_2O_{dest.}$, store sterile in 1 ml fractions.

Preparation solution pH 7.2

GBSS	98ml
Glucose	1 ml
optional (not for dissociated cultures)	
Kynurenic acid	1 ml

Medium

BME	100 ml
HBSS	50 ml
Equine donor serum	50 ml
GlutaMAX (200 mM)	1 ml
Glucose (50%)	2 ml

Dissociated cultures:

Medium dissociated cultures

Neurobasal	50 ml
B27	2 ml
GlutaMAX (200 mM)	125 µl

Borate-Buffer pH 8.5 (Glass coverslips)

Dissolve 1.24 g boric acid and 1.9 g borax in 400 ml $H_2O_{dest.,}$ adjust pH to 8.5

Cell and Molecular Biology:

Phosphate buffered saline (PBS)

COMPONENT	MOLARITY (mM)
KCl	2.7
KH_2PO_4	1.5
NaCl	137
Na_2HPO_4	10.4

Phosphate buffer (0.2 M, pH 7.4)

COMPONENT	MOLARITY (mM)
$NaH_{2PO}4*2H_2O$	0.04
$Na_2HPO_4*2H_2O$	0.17

Lyses buffer for purification of genomic DNA from tails

COMPONENT	MOLARITY (mM)
Tris/HCl pH 8	100
NaCl	200
EDTA	5
SDS	0.2%
Proteinase K	100 µg/ml

3.3 Cell culture techniques

3.3.1 Preparation of organotypic hippocampal cultures

Organotypic hippocampal slice cultures were prepared as previously described (Stoppini et al., 1991). Mice p5/6 were rapidly decapitated, the skull removed and the dorsal half of the brain was transferred to ice cold GBSS. 400 µm transversal hippocampal slices were cut using a McIllwain tissue chopper and kept at 4 °C for 30 min in GBSS. Subsequent cultivation was performed on tissue culture inserts (Millicell, 0.4 µm pore size, Hydrophilic PTFE membrane), 4 slices each insert, at 37 °C, 5% CO_2 and 99% humidity. To reduce the number of non neuronal cells, antimitotic drugs (uridine, cytosine-β-D-arabinofuranoside* hydrochloride and 5-fluoro-2´-deoxyuridine) were applied for 24 h three days after preparation. Subsequently, 50% medium was changed every 3 d.

3.3.2 Preparation of dissociated cultures

Primary cultures of mouse hippocampal neurons were prepared using mice (C57 Bl/ 6) at embryonic day E 18. Embryos were decapitated and the brains kept in ice cold Gey's balanced salt solution supplemented with glucose. Tissue was dissociated by 30 min incubation in trypsin/ EDTA at 37 °C followed by mechanical dissociation using a Pasteur pipette. Cells were plated at high density (10^5) on poly-L-lysine coated cover slips (13 mm) and kept in Neurobasal medium (Gibco) supplemented with 2% B27 (Gibco) and 0.5 mM Glutamax at 37 °C, 5% CO_2 and 99% humidity.Cell culture medium was not changed.

Glass coverslips were treated with 10 M NaOH (5-6 h), sterilized at 225 °C for 6 h and coated with 0.5 mg/ ml poly-L-lysine (Sigma) in boric acid buffer (2-3 h at 37 °C).

3.4 Transfection of hippocampal neurons

3.4.1 Biolistic Transfection using the Helios Gene Gun

Organotypic hippocampal slice cultures were transfected at various time points (7 DIV for shPFNIIa, 12 DIV for PFNIIa overexpression and 14 DIV for the experiments with neurotrophin receptors) using the Helios Gene Gun system of Bio-Rad. Gold microcarriers 600 nm in diameter were shot onto the slice with helium at a pressure of 100 psi. To avoid damage of the tissue due to gold clumps, culture inserts with a pore diameter of 3 µm were used as filters (Figure 6).

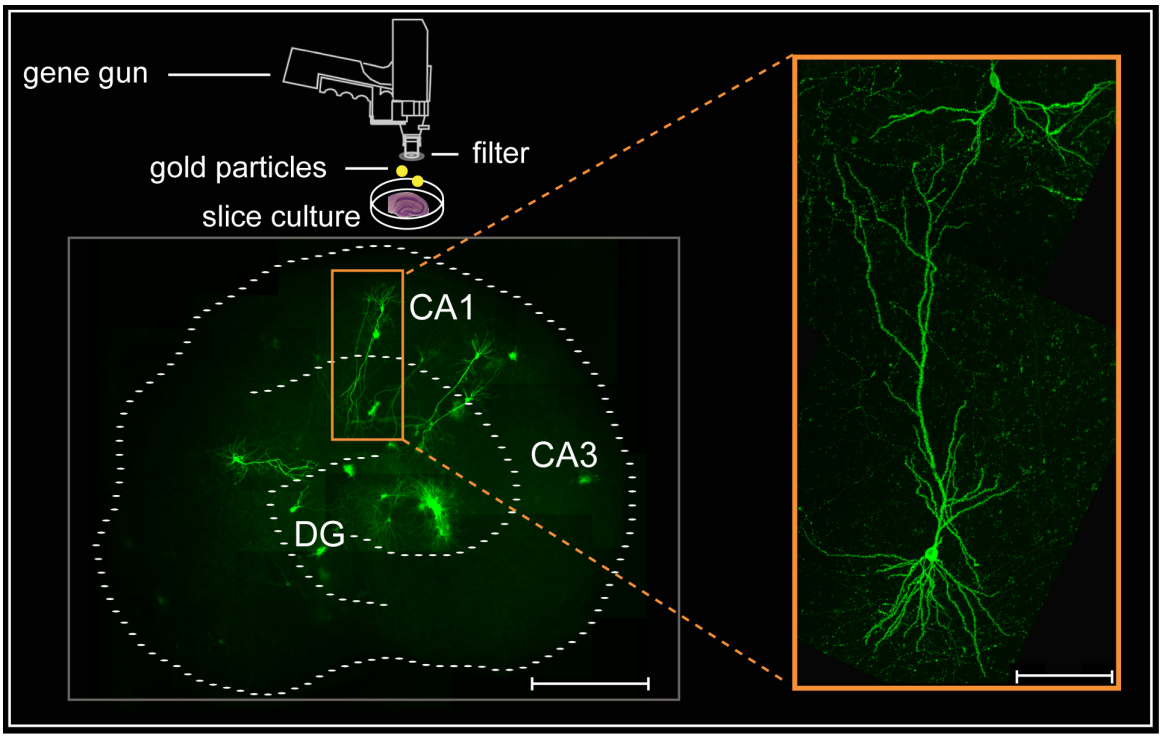


Figure 6 | Biolistic transfection of organotypic hippocampal slice cultures using the Helios Gene Gun (Bio-Rad)
Gold particles coated with *fGFP* were shot onto the slice; all principal neurons of the hippocampus can be transfected; RIGHT: 20x image of a CA1 pyramidal neuron and a granule cell of the dentate gyrus (top); , scale bar (left) 500 µm, scale bar (right) 100 µm.

Bullets for transfection were prepared at least one day in advance using the tubing preparation station provided with the Gene Gun. For one plasmid 12.5 mg of gold microcarriers and 25 µg of DNA were used, for two plasmids 15 mg of gold and 30 µg of DNA were prepared. To visualize neuronal morphology in detail, a membrane targeted form of eGFP (fGFP*)* was transfected at a ratio of 1:2. For the coating of DNA onto the

gold microcarriers Ca_2Cl precipitation was performed (Wellmann et al., 1999;O'Brien and Lummis, 2006). Briefly, the gold microcarriers were mixed with 100 µl of 0.05 M spermidine (Sigma) and clumps were destroyed by 10 s sonication. Fresh spermidine solution was prepared at least every month and stored at 4 °C. 100 µl of 1 M Ca_2Cl were added drop-wise followed by 10 min incubation at room temperature. To clean the gold microcarriers of residual spermidine four washing steps in 96% ethanol were performed using a microcentrifuge at 100 g. Finally, the gold microcarriers were dissolved in 1 ml of 96% ethanol containing 0.05 mg/ml polyvinylpyrrolidone (PVP). 75 cm of Tefzel tubing (Biorad) were cleaned by 10 min flow on nitrogen in the tubing preparation station (Biorad) and subsequently the gold suspension was inserted. To ensure homogenous distribution of gold the tubing was rotated 30 s and then dried with nitrogen for 5 min.

3.4.2 Transfection of primary hippocampal cultures

Primary cultures of hippocampal neurons made E 18 were transfected at various time points using Lipofectamine2000® following manufactures instructions. Briefly, half of the culture medium was exchanged one day prior to transfection and the old medium was collected and kept at 4 °C. 0.8 µg DNA as well as 2 µl Lipofectamine per well were diluted in Neurobasal medium (incubation for 5 min), subsequently combined (incubation for 20 min) and added to the cultures drop-wise. Medium was exchanged after 50 min giving back the medium of the day before substituted with 50% new medium.

3.5 Immunocytochemistry

Organotypic as well as dissociated hippocampal cultures were fixed over night at 4 °C with 4% paraformaldehyde in 0.1 M phosphate buffer. Blocking and permeabilization were performed for 1 h at room temperature in phosphate buffered saline containing 1% BSA, 10% goat serum and 0.2% Triton X-100. All primary antibodies were incubated at 4 °C. Rabbit polyclonal anti-human $p75^{NTR}$ (Promega) was used at a dilution of 1:500 (3 d) for organotypic cultures and 1:4000 (overnight) on dissociated neurons. Monoclonal mouse anti-PFNIIa antibody (Murk K et al., 2009) was diluted 1:100 for organotypic cultures (7 d) and 1:200 on dissociated neurons (overnight). Polyclonal rabbit anti-T1 antibody (Santa Cruz, directed against the intracellular domain specific for T1) was diluted 1:1000 (overnight) for dissociated neurons. Secondary anti mouse or rabbit

antibodies conjugated with Cy2, Cy3 or Cy5 (Jackson ImmunoResearch) were incubated 1:500 in BPS for 2 h at room temperature.

3.6 Image acquisition and analysis

Neurons were imaged using an Axioplan 2 microscope (Zeiss) equipped with an Apotome® (Zeiss) controlled by the Axiovision® software. To image the entire CA1 neurons in the organotypic cultures several z-stacks of 1 µm were acquired using a 20x 0.8 NA Plan-APO objective (Zeiss). In dissociated cultures plain fluorescence images of hippocampal neurons were acquired. For analysis of spine density in organotypic cultures parts of basal and both proximal and distal apical dendrites were imaged at a higher magnification with a 63x 1.4 NA Plan-APO oil immersion objective (Zeiss) and a z-stack thickness of 0.5 µm (Figure 7). In dissociated neurons spines of secondary as well as tertiary dendrites in the mid part of the dendritic tree were imaged using the same settings as above.

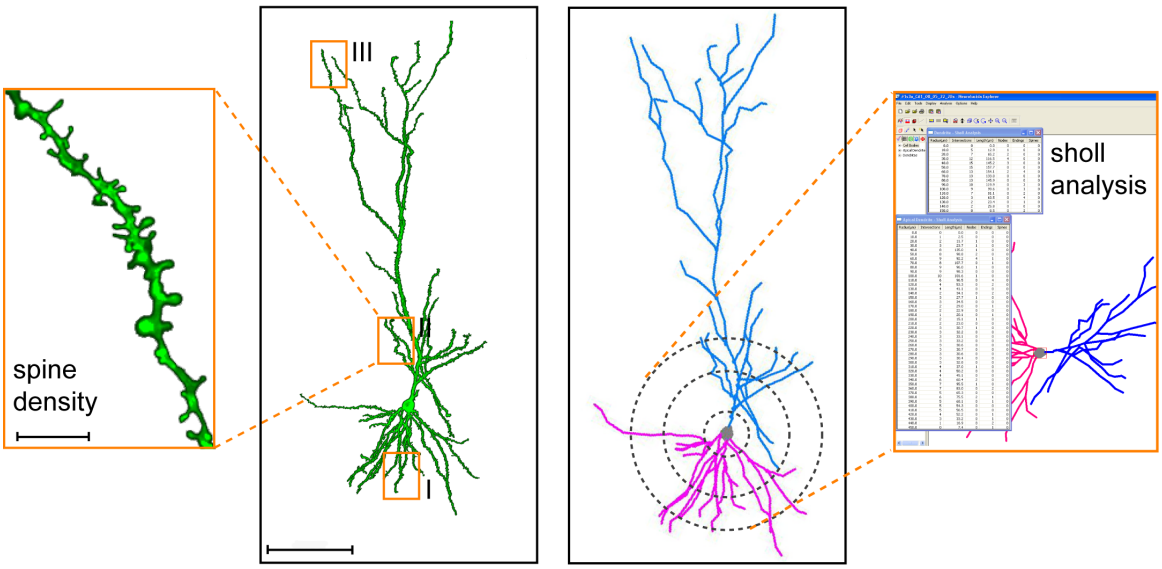


Figure 7 | Morphological analysis of pyramidal neurons in the mouse hippocampus
LEFT, CA1 pyramidal cell captured from a maximum intensity projection, scale bar 100 µm, the three different regions for spine density counts are labeled, spine density is counted on higher magnification images, scale bar 5 µm. RIGHT, Neurolucida® representation (tracing) of the CA1 pyramidal neuron shown at the left, dotted circles indicate Sholl analysis perfomed with Neurolucida Explorer®.

The knockdown of PFNIIa was quantified by using a Zeiss 510 META confocal microscope with a 40 X 1.3 NA oil immersion objective and zoom of 2. Mean pixel intensity of the neuronal soma was measured for shPFNIIa transfected cells (18 neurons

of 3 independent experiments) and untransfected neighbour neurons (18 neurons of 3 independent experiments) stained with anti-PFNIIa. Average pixel intensity of untransfected cells was set to 100%.

Morphological analysis was performed using Neurolucida® and Neurolucida Explorer® software (Microbrightfield) (Figure 7). Briefly, for Sholl analysis the program uses an algorithm setting a series of concentric circles around the neuronal soma, thereby counting how many neurites cross each circle (Figure 7). Obtained values for Sholl analysis (Sholl, 1953), spine density or dendrite number and length were exported to Excel and Graphpad prism for statistical analysis using a paired student's t-test (two-tailed and two-sample unequal variance); significance was set at $p < 0.05$. For the Sholl analysis data significance was only considered if more than two adjacent points showed p values below 0.05. All data are shown as mean + SEM

3.7 **Mice strains**

Transgenic mice expressing TrkB.TK+ or TrkB.T1 under the Thy 1.2 promoter were generated in the group of Eero Castren (University of Helsinki, Figure 8) (Saarelainen et al., 2000b;Koponen et al., 2004a;Koponen et al., 2004b).

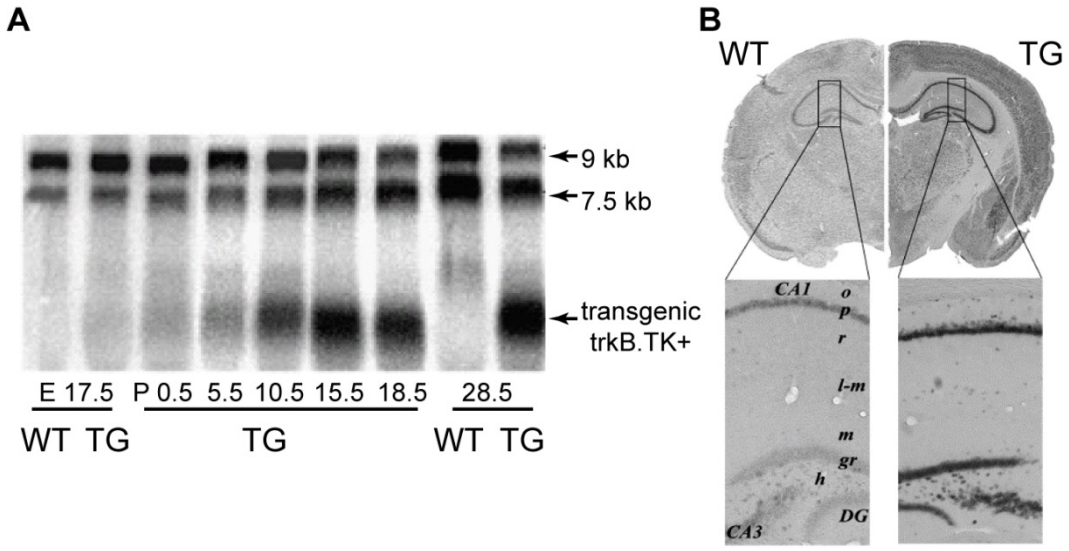


Figure 8 | Transgenic mice overexpressing TrkB.TK+
A, Northern blot showing the overexpression of the full-length TrkB neurotrophin receptor under the Thy 1.2 promoter at various time points against the endogenous trkB.TK+ levels, (adapted from Koponen et al. 2004b); **B**, in-situ hybridization showing increased mRNA expression in the hippocampus for the full-length isoform in transgenic mice overexpressing TrkB.TK+, abbreviations: TG, transgenic; o, stratum oriens, p, st. pyramidale, l-m, st. lacunosum-moleculare; m, st. molecular; gr, granule cell layer; h, hilus; DG, dentate gyrus; (taken from Koponen et al. 2004a)

Briefly, cDNAs were tagged N-terminally with an eight amino acid FLAG peptide and inserted into the murine Thy 1.2 expression cassette to direct expression to postnatal neurons. The constructs were transferred by pronucleus injection into embryos from CD2F1 female (BALB/c x DBA/2) mated with CD2F1 males.

Expression of the transgene starts at p10, increases until p18 and remains stable thereafter (Koponen et al., 2004b) (Figure 8). The transgenic expression of TK+ corresponds to a four-fold increase compared to the endogenous expression levels (Koponen et al., 2004b), whereas the overexpression of T1 reaches a level of about 20-fold of the endogenous mRNA (Saarelainen et al., 2000a). Strong expression of the transgene can be detected in hippocampal pyramidal neurons, dentate granule cells and pyramidal neurons of the cerebral cortex (Koponen et al., 2004b) (Figure 8).

For all experiments animals were genotyped by PCR using genomic DNA extracted from tail pieces (for primers and PCR conditions see in Table 1 and Table 2). CD2F1 mice

were used as WT controls for experiments with TrkB.TK+ and TrkB.T1 transgenic animals. All other experiments were done using cultures of C57BL/ 6 mice.

3.8 Molecular biology

All molecular work and preparation of media was done as described elsewhere (Sambrook et al., 1989). The bacterial strains used for this study are *E. coli* DH5 or DH10.

3.8.1 Genotyping of transgenic mice

Genomic DNA was extracted from tail pieces. Briefly, tails were digested over night in lyses buffer at 55 °C. Cellular debris was removed by centrifugation at 14.000 g. Genomic DNA was precipitated using isopropanol and washed once using 70% ethanol. The DNA was stored at -20 °C in 10 mM Tris/ HCl (pH 8).

PCR was performed with *thy 1.2* and *trkB* specific primers (for primers see Table 1, for PCR conditions see Table 2). The resultant PCR product has a size of 500 bp. It is not possible to distinguish between TrkB.TK+ and TrkB.T1 using this PCR.

Table 1 | Primer for genotyping of TrkB.TK+ and TrkB.T1 transgenic mice

primer sequence		Reference
TrkB forw.	CTCCCACTTCCTTGGCTT *(thy 1.2 region)*	(Koponen et al., 2004b)
TrkB. rev.	GCCCCACGTAAGCTTCGA *(trkB gene)*	(Koponen et al., 2004b)

Table 2 | PCR protocol for genotyping of TrkB.TK+ and TrkB.T1 transgenic mice

step	temperature	time
1 ***denaturation***	95 °C	1 min
2 ***denaturation***	95 °C	30 s
3 ***annealing***	54 °C	1 min
4 ***synthesis***	72 °C	1 min
5 ***repeat***	steps 2-4	34 times
6 ***endsynthesis***	72 °C	7 min

3.8.2 Preparation of DNA

The purification of plasmid DNA for transfection of hippocampal neurons was done using MAXI or MIDI plasmid purification kits (Qiagen). An overview about the plasmids used in this work can be seen in Table 3.

Table 3 | Plasmids used in this study

plasmid	description	reference
fGFP	farnesylated enhanced green fluorescent protein (CMV promoter)	Clontech
fcherry	farnesylated fluorescent protein mcherry (CMV promoter)	(Shaner et al., 2004;O'Brien, 2007)
p75^{NTR}	mouse p75 neurotrophin receptor (CMV promoter)	(Zagrebelsky et al., 2005)
T1	HA-tagged rat TrkB.T1 (CMV promoter)	(Haapasalo et al., 1999)
T1-EC	FLAG-tagged rat TrkB.T1 with a deletion of the extracellular domain (EF-1α promoter)	(Haapasalo et al., 1999)
T1-IC	FLAG-tagged rat TrkB.T1 with a deletion of the T1-specific domain (EF-1α promoter)	(Haapasalo et al., 1999)
PFNIIa	mouse profilinIIa (truncated CMV promoter)	(Boshart et al., 1985;Murk K, 2008)
shPFNIIa	polycistronic vector: 1. profilinIIa-specific shRNA sequence (CMV/U6.3 promoter), 2. reporter fGFP (CMV promoter)	(Murk K, 2008)
shPFNIIa-mod	polycistronic vector: 1. profilinIIa-specific shRNA sequence (CMV/U6.3 promoter), 2. RNAi-resistant modified profilinIIa (truncated CMV promoter)	(Murk K, 2008)
shPFNIIa R74E	polycistronic vector: 1. profilinIIa-specific shRNA sequence (CMV/U6.3 promoter), 2. RNAi resistant modified profilinIIa impaired in actin binding (truncated CMV promoter)	(Murk K, 2008)
shPFNIIa Y29,133S	polycistronic vector: 1. profilinIIa-specific shRNA sequence (CMV/U6.3 promoter), 2. RNAi resistant modified profilinIIa impaired in poly-L-proline binding (truncated CMV promoter)	(Murk K, 2008)

4 RESULTS

4.1 The expression levels of neurotrophin receptors modulate neuronal morphology

The diversity of cell-biological functions mediated by neurotrophins results from their interaction with two distinct types of receptors: the tropomyosin-related kinase receptors (Trk) and the pan neurotrophin receptor $p75^{NTR}$. Interestingly, these distinct types of neurotrophin receptors have been shown to mediate often opposing effects as neuronal survival (mediated by Trk signaling) or apoptosis (mediated by $p75^{NTR}$) (for reviews see Huang and Reichardt, 2001;Dechant and Barde, 2002;Huang and Reichardt, 2003;Teng and Hempstead, 2004;Blochl and Blochl, 2007). In addition, $p75^{NTR}$ can enhance Trk signaling (reviewed in Huang and Reichardt, 2001;Chao, 2003).

In the first part of my work, I addressed the question how alterations in the expression level or the activation of neurotrophin receptors could affect the morphology of mature CA1 pyramidal neurons. By this means, I was able to investigate how the ratio of neurotrophin receptor expression levels determines the cellular consequences of neurotrophin action.

4.1.1 $p75^{NTR}$ is a negative modulator of neuronal morphology

Particle mediated gene transfer (Lo et al., 1994;O'Brien and Lummis, 2006) was used to transfect individual pyramidal cells of organotypic hippocampal slice cultures. Due to the low transfection efficiency of the gene gun method, only few isolated cells are normally transfected in each slice culture. The expression of fGFP (a membrane targeted form of eGFP) led to the intense labelling of the entire dendritic tree of each neuron. Moreover, the fine structure of dendritic protrusions as spines could be observed without toxic side effects (Nakayama et al., 2000;Zagrebelsky et al., 2005). All principle cell types of the hippocampus – CA1, CA3 and granule cells – were transfected. In addition, glia cells and interneurons could as well be observed.

In the first series of experiments, fully developed pyramidal neurons of organotypic hippocampal slice cultures were transfected with $p75^{NTR}$ and fGFP at 14 DIV (De Simoni

et al., 2003) and fixed 3 days later. P75^{NTR} overexpression in CA1 pyramidal neurons was confirmed via immunocytochemistry using anti-p75^{NTR} antibody (Promega) (Figure 17).

Mature CA1 pyramidal neurons are characterized by two distinct and highly branched dendritic trees. Several basal dendrites emerge from the base of the neuronal soma, whereas a single apical dendrite with various oblique branches emanates from the apex. The branching patterns of these dendritic compartments (basal and apical) can be analyzed in detail using the Sholl analysis method (Sholl, 1953).The number of dendritic branches (quantified as the number of intersections with consecutive, concentric circles centred at the neuronal soma) is therefore plotted in relation to their distance from the neuronal soma.

The apical dendrites of CA1 pyramidal neurons transfected with fGFP and p75^{NTR} showed a significant reduction in dendritic complexity compared to control cells transfected with fGFP only (Figure 9A apical). Negative morphological changes were restricted to the proximal part of the apical dendrites. This part of the apical dendritic tree of neurons in the hippocampus is located in the stratum radiatum, which is innervated via the Schaffer collaterals from the CA3 region. Interestingly, the basal dendrites, too, receive input via the Schaffer collaterals, however, dendritic complexity of this compartment was unaltered in p75^{NTR} overexpressing neurons compared to control cells (Figure 9A basal).

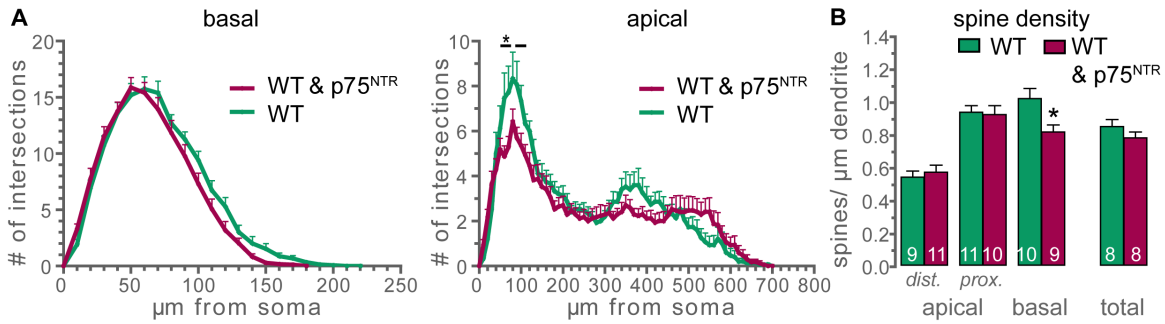


Figure 9 | The Overexpression of p75^{NTR} negatively modulates the morphology of CA1 pyramidal neurons.
A, Sholl analysis (basal and apical dendrites) of CA1 neurons in organotypic hippocampal slice cultures (17 DIV) overexpressing p75^{NTR} (n=17) for 3 days compared to control cells (n=15), neurons overexpressing p75^{NTR} show a significant reduction in dendritic complexity of the apical dendrites. **B**, spine density analysis of p75^{NTR} overexpressing cells compared to control neurons reveals a significant reduction in spine number of the basal dendritic compartment; *p < 0.05.

In a next step, spine density counts were performed for the different parts of the dendritic tree, basal dendrites as well as proximal and distal apical dendrites. Spine density of CA1 neurons is inhomogeneous along their dendritic tree (Figure 9B), with higher spine

numbers in the proximal parts and less dendritic protrusions in distal regions. This is consistent with the distribution of excitatory synapses on CA1 neurons already described *in vivo* (Megias et al., 2001). Remarkably, the basal dendrites of $p75^{NTR}$ overexpressing cells showed significantly less spines compared to control neurons. Yet, spine density of the other dendritic compartments was unaffected (Figure 9B; spine numbers and corresponding p values can be seen in the supplement Table S1). These observations are in line with an already described role of $p75^{NTR}$ as a negative regulator both of dendritic complexity as well as spine density (Zagrebelsky et al., 2005). Interestingly, the overexpression of $p75^{NTR}$ affected either the number of dendritic branches or the number of spines depending on the dendritic compartment (basal versus apical dendrites) analyzed.

Neurotrophins have been suggested to be involved in the modulation of synaptic transmission. BDNF and its receptor TrkB have been shown to be crucial for the long-lasting enhancement of synaptic efficacy (long-term potentiation, LTP). In contrast to this, studies in $p75^{NTR}$ knockout mice suggest an important role of this receptor for the opposite form of synaptic plasticity. Specifically, low frequency stimulation of the Schaffer collaterals in hippocampal slices has been shown to induce a long-lasting decrease in synaptic efficacy (long-term depression, LTD). LTD maintenance is significantly impaired in $p75^{NTR}$ knockout mice (Rosch et al., 2005;Woo et al., 2005). Moreover, the application of pro-BDNF, which binds preferentially to $p75^{NTR}$, was reported to enhance LTD. This facilitation could be blocked by application of a $p75^{NTR}$ antibody suggesting that the activation of $p75^{NTR}$ is crucial for LTD maintenance (Woo et al. 2005). In this context $p75^{NTR}$ could provide a link between activity-dependent synaptic plasticity and subsequent negative changes in neuronal morphology.

In order to investigate morphological changes following the activation of $p75^{NTR}$ in mature CA1 pyramidal neurons, a brief bath application of NMDA (N-methyl-D-aspartic acid; 20 µM, 10 min) was used (Figure 10). NMDA is a specific agonist of the NMDA-type of glutamate receptors. Bath application of glutamate or NMDA has been shown to stimulate extrasynaptic as well as synaptic NMDA receptors, which in turn could lead to neuronal cell death because of excitotoxicity (Rothman and Olney, 1995;Hardingham et al., 2002). Therefore, a low concentration of NMDA was used (20 µM) that has been shown before to activate predominantly synaptic NMDA receptors (Soriano et al., 2006) and moreover to induce $p75^{NTR}$-mediated LTD (Woo et al., 2005) (Figure 10). Hippocampal slice cultures were fixed 48 h after NMDA stimulation (17 DIV).

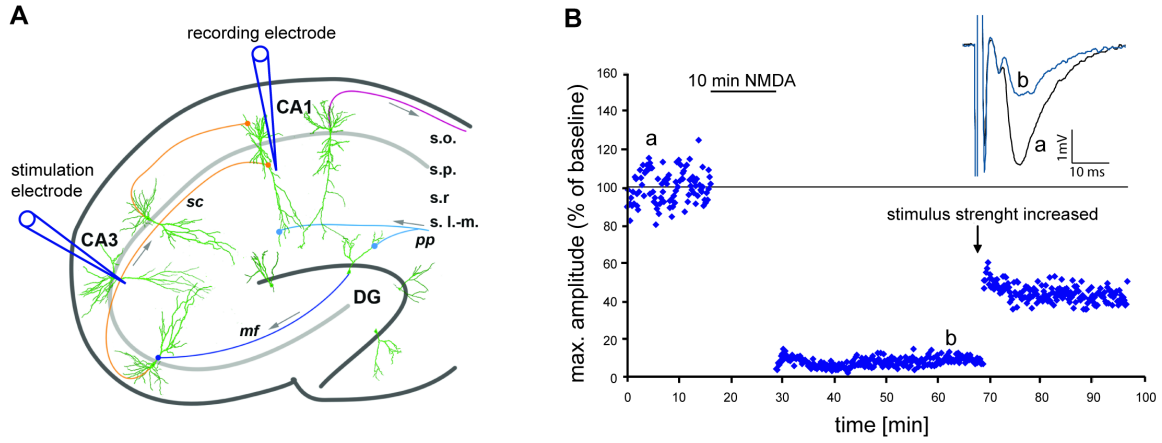


Figure 10 | Chemical induction of long-term depression via the application of NMDA
A, Recording of field excitatory postsynaptic potentials (fEPSPs) was done with a microelectrode positioned in the stratum radiatum of the CA1 area, the stimulus electrode was positioned in the CA3 region (for details see Korte et al. 1995a). **B**, the application of 20 µM NMDA for 10 min leads to a long lasting decrease in synaptic efficacy, sample traces are shown at indicated time points labeled a and b.

An example of LTD induced in an organotypic hippocampal slice culture by the application of NMDA can be seen in Figure 10. Electrophysiological recording was done by V. Staiger and M. Korte.

The overall dendrite structure of NMDA treated neurons was normal compared to control cells, suggesting that the stimulation indeed did not lead to any degeneration of CA1 cells. Sholl analysis of basal and apical dendrites revealed only a slight non significant decrease in dendritic complexity of NMDA treated neurons compared to control cells, which was restricted to the basal dendrites (Figure 11A).

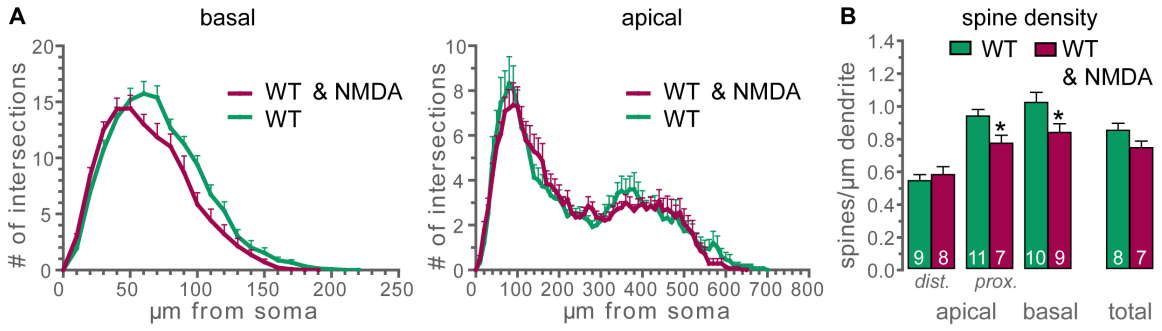


Figure 11 | The chemical induction of long-term depression significantly decreases spine density of CA1 neurons.
A, Sholl analysis (basal and apical dendrites) of CA1 neurons in organotypic hippocampal slice cultures (17 DIV) 48 h after the chemical induction of long-term depression (LTD) (n=14) via a 10 min application of 20 µM NMDA; the induction of LTD leads to a slight, non significant decrease of dendritic complexity of the basal dendrites. **B**, spine density of CA1 neurons after LTD induction, LTD significantly decreases the number of spines both of the basal dendrites as well as of the proximal apical dendritic compartment; *p < 0.05.

Interestingly, the chemical induction of LTD resulted in the loss of spines both in the basal dendrites as well as in the proximal apical dendrites as spine density counts revealed a significant reduction for both compartments (Figure 11B, supplement Table S1).

Time-lapse imaging studies already showed that spines can undergo bidirectional activity-dependent morphological plasticity (Nagerl et al., 2004;Zhou et al., 2004). Growth of new spines could be observed after high frequency stimulation, whereas a protocol using low-frequency stimulation induced the pruning of already existing spines (Nagerl et al., 2004). The observation of a reduced number of dendritic protrusions following the chemical induction of LTD in the current study further supports the hypothesis that functional changes in synaptic efficacy can be translated into structural changes.

In summary, these results demonstrate that both the overexpression of $p75^{NTR}$ as well as the activation of this receptor negatively regulate neuronal morphology. These observations therefore further promote a role of $p75^{NTR}$ as a negative modulator of neuronal structure. Interestingly, negative structural changes of the two dendritic compartments (basal and apical dendrites) were found to be restricted to either dendrites or spines.

4.1.2 Overexpression of TrkB receptor splice variants alters the morphology of CA1 pyramidal neurons

In contrast to $p75^{NTR}$ the Trk receptors primarily exert well-defined trophic functions (reviewed in Reichardt, 2006). Especially TrkB is known to enhance axonal as well as dendrite growth (reviewed in McAllister et al., 1999). BDNF signaling via TrkB has also been shown to be crucial for LTP (Korte et al., 1995a) and to positively modulate spine density (Tyler and Pozzo-Miller, 2001).

Interestingly, this receptor occurs in different splice variants: the full-length receptor (TrkB.TK+), which carries the signal transducing kinase domain and two truncated receptors lacking this domain (TrkB.T1 and TrkB.T2) (Klein et al., 1990;Middlemas et al., 1991). Whereas TK+ is the predominant isoform early in development, T1 expression is upregulated at postnatal stages and can even exceed the expression of TK+. Despite the striking change in expression levels of TK+ and T1 not much is known about the role of the truncated receptor in the central nervous system.

In order to study the role of full-length TK+ and truncated T1 in regulating the morphology of mature CA1 pyramidal neurons, organotypic hippocampal cultures of

transgenic mice expressing either one of them under the Thy1.2 promoter were used (Saarelainen et al., 2000a;Koponen et al., 2004b). Expression of the transgene starts at p10, increases until p18 and remains stable thereafter (Koponen et al., 2004b). The transgenic expression of TK+ corresponds to a four-fold increase compared to the endogenous expression levels (Koponen et al., 2004b), whereas the overexpression of T1 reaches a level of about 20-fold of the endogenous mRNA (Saarelainen et al., 2000a). Strong expression of the transgene can be detected in hippocampal pyramidal neurons, dentate granule cells and pyramidal neurons of the cerebral cortex (Koponen et al., 2004b).

For detailed morphological analysis, hippocampal slice cultures of transgenic TK+ animals were transfected with fGFP using particle-mediated gene transfer at 14 DIV and fixed 72 h later.

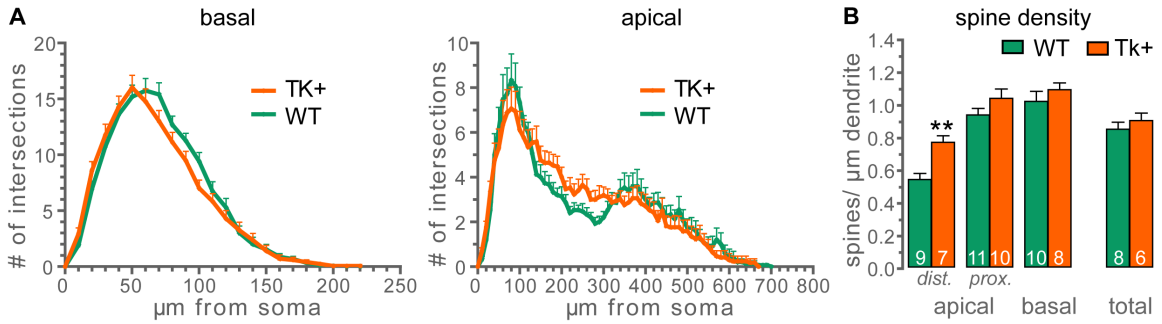


Figure 12 | The overexpression of full-length TrkB increases spine density of CA1 neurons.
A, Sholl analysis (basal and apical dendrites) of CA1 neurons in transgenic organotypic hippocampal slice cultures (17 DIV) overexpressing the full-length TrkB (TK+) receptor (n=15) compared to control cells (n=15); the overexpression of TK+ only slightly increases dendritic complexity. **B**, spine density of CA1 neurons overexpressing TK+; the transgenic overexpression of TK+ significantly increases the number of spines of the distal apical dendrites compare to WT neurons; **p < 0.005.

Neurons overexpressing TK+ showed an overall normal neuronal morphology compared to control cells (Figure 12A). Detailed Sholl analysis revealed only a slight and non significant increase in dendritic complexity of the apical dendrites (Figure 12A). The number of dendritic spines was increased in cells overexpressing TK+ in all dendritic compartments. However, a significant increase in spine density could only be detected in the distal apical tufts of CA1 pyramidal neurons (Figure 12B, supplement Table S1). This part of the dendritic tree is located in the stratum lacunosum-molecular and receives input from the entorhinal cortex (Figure 2). Notably, in organotypic hippocampal slice cultures the connection to the entorhinal cortex is disrupted. In contrast to this, the proximal part of the apical dendrite as well as the basal dendrites are innervated by the CA3 region.

Mature CA1 neurons overexpressing T1 showed a marked simplification of the mid-apical dendrite (Figure 13A arrows). In addition, the apical dendrite displayed a striking elongation (Figure 13A).

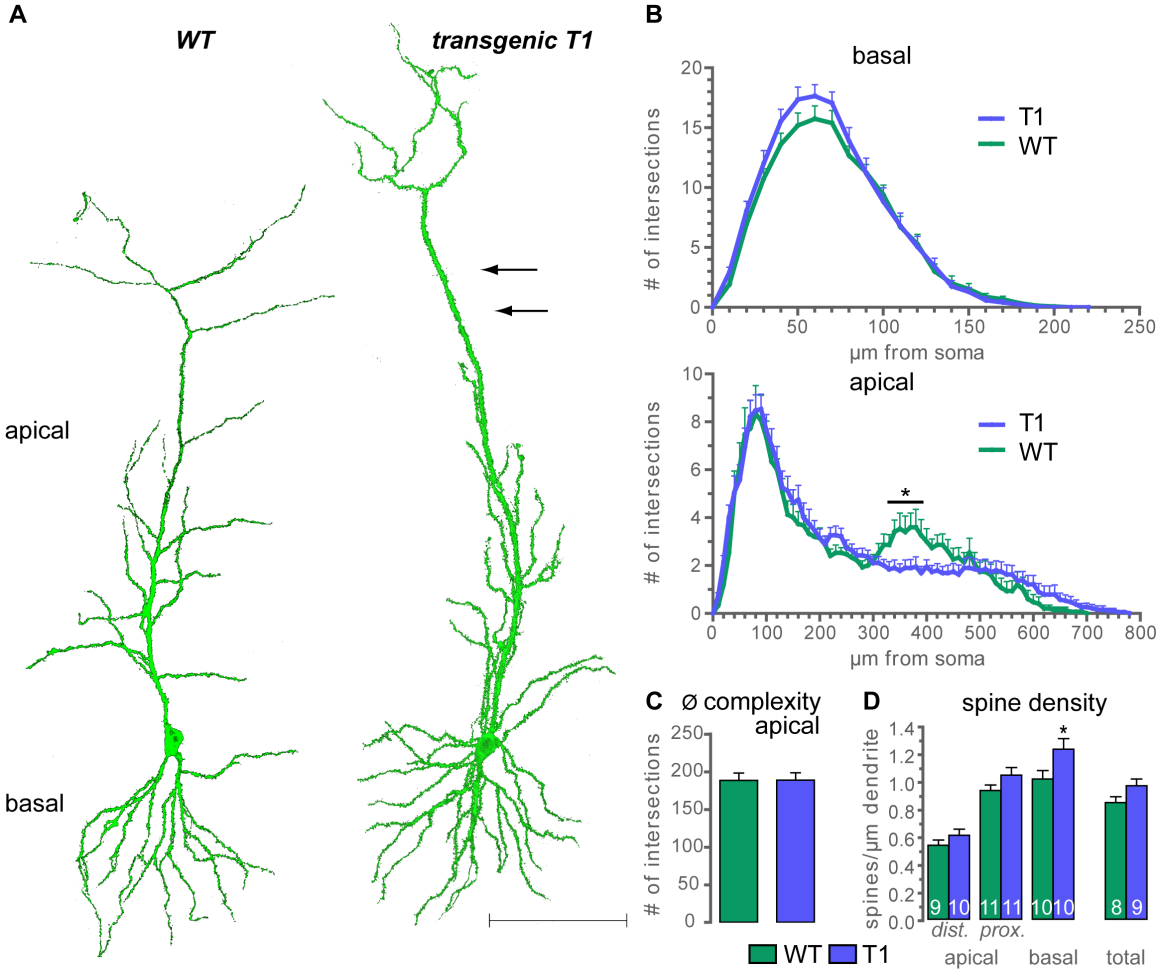


Figure 13 | The overexpression of the truncated TrkB receptor splice-variant alters neuronal morphology.
A, CA1 neurons (17 DIV) of transgenic mice overexpressing TrkB.T1 (T1) compared to WT cells, T1 overexpressing cells show a reduction in dendritic complexity of the mid-apical dendrite (arrows) compared to control cells; images were captured from maximum intensity projections; scale bar 100 µm. **B**, Sholl analysis (basal and apical dendrites) of T1 overexpressing CA1 neurons (n=17) compared to control cells (n=15), the overexpression of T1 significantly decreases dendritic complexity of the apical dendrite at a distance between 330 and 430 µm from soma, in addition, T1 overexpressing neurons are longer than WT cells. **C,** total dendritic complexity and **D**, spine density of CA1 pyramidal neurons overexpressing T1; the overexpression of T1 significantly increases the spine number of the basal compartment, whereas the total dendritic complexity is unaltered in T1 overexpressing neurons compared to control cells; *$p < 0.05$.

Detailed Sholl analysis of T1 overexpressing neurons revealed a slight but non significant increase in dendritic complexity of the basal dendrites compared to control cells (Figure 13B). In contrast to this, dendritic complexity of the apical dendrites was significantly reduced at a distance between 330 and 430 µm from the soma (Figure 13B). Interestingly, morphological alterations were restricted the distal half of the apical dendrite: a decrease

in complexity of the mid-apical compartment and an elongation of the apical tufts. The proximal apical dendrites, however, showed no alterations compared to control cells. Notably, total dendritic complexity of the apical dendrite was found to be unaltered compared to control cells, as the decrease in complexity and the simultaneous elongation of the distal tufts outweighed each other (Figure 13C). As a next step, spine density counts were performed on T1 overexpressing CA1 neurons. Total spine density was increased in T1 overexpressing cells (Figure 13D, supplement Table S1). However, a significantly increased number of dendritic protrusions could be shown only for the basal dendritic compartment (Figure 13D). Again, as in the case of the overexpression of $p75^{NTR}$, changes on the level of spines and on the level of dendrites were restricted to different neuronal compartments (basal versus apical dendrites).

Spines have been shown to undergo activity-dependent structural remodeling (Engert and Bonhoeffer, 1999;Maletic-Savatic et al., 1999). Therefore it is interesting to see if changes in spine density could indeed be correlated to changes in spine shape. By means of morphological analysis three different spine subtypes can be classified: stubby, thin and mushroom spines (Figure 14A) (Chicurel and Harris, 1992;Koh et al., 2002). This classification was set up by measurements of the total spine length as well as the ratio between the diameters of the spine head and the spine neck (Figure 14B).

Stubby spines therefore are shorter than 1 µm and lack a clear spine head. The prototype of a spine (mushroom) shows a bulbous head which can be clearly discriminated from the narrower neck (Figure 14A, B). Mushroom spines are thought to carry functional synapses and changes in the size of their heads have been shown to be correlated to functional changes in synaptic strength (for reviews see Hering and Sheng, 2001;Cingolani and Goda, 2008). Thin spines are longer than 1 µm and carry only a small head (Figure 14A, B). They are reported to be highly dynamic. Interestingly, thin spines have been shown to retract or to develop into mushroom spines according to changes in synaptic activity (Parnass et al., 2000;Trachtenberg et al., 2002).

In line with previous reports, mushroom spines comprised the predominant spine type in mature pyramidal neurons analyzed in this study (Zagrebelsky et al., 2005;Chakravarthy et al., 2006). Interestingly, T1 overexpressing neurons showed no alterations in spine subtype composition (Figure 14B) in the basal and in the proximal apical compartment.

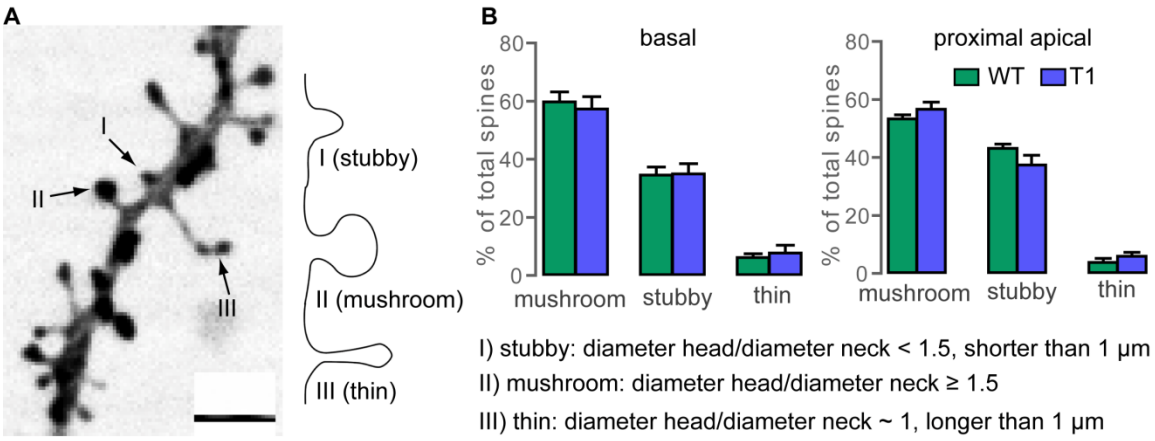


Figure 14 | Spine subtype composition is not altered in CA1 neurons overexpressing TrkB.T1.
A, representative image of a dendrite from a WT neuron showing the three spine types, scale bar 2 µm; spine types can also be seen in the illustration at the right (criteria used for classification are indicated in B based on the length of the spine and the ratio of the diameters of the head and neck): type I stubby spine, type II muhroom spine and type III thin spine. **B**, proportion of the three spine types (basal as well as proximal apical dendrites) of T1 overexpressing CA1 neurons (n=5) compared to control cells (n=5), spine subtype composition is not affected by the overexpression of T1.

These observations suggest that indeed the increase in the number of spines in the basal compartment following T1 overexpression (Figure 13B) might be correlated to an increase in the number of synapses in these neurons. However, colocalization studies with synaptic markers as synapsin or PSD95 still need to proof this assumption.

In order to investigate if morphological alterations due to the overexpression of TrkB receptor isoforms were restricted to CA1 pyramidal neurons, dendritic complexity of the other principle cell types of the hippocampus was analyzed (Figure 15A). Pyramidal neurons of the CA1 and CA3 region vary considerable with respect to their dendritic architecture, gene expression, spine structure or membrane properties (Lein et al., 2004;for reviews see Migliore and Shepherd, 2005;Spruston, 2008). Interestingly, TK+ overexpressing CA3 neurons showed a significant decrease in dendritic complexity of the apical dendrites compared to control cells (Figure 15B). Notably, this phenotype is opposite to the one observed following TK+ overexpression in CA1 neurons (Figure 12). The overexpression of T1 in CA3 neurons induced a slight but non significant reduction in the complexity of the apical dendrites. Basal dendrites were found to be unaltered by overexpression of both receptor types compared to control cells (Figure 15B).

Granule cells of the dentate gyrus constitute the third type of excitatory neurons in the hippocampus (Figure 15A, C). They are different from the neurons described before as they are not pyramidal in morphology. Granule cells have a small, ovoid cell body with a small dendritic tree that is – as the one of pyramidal neurons – studded with spines

(Figure 15C). Dentate granule cells receive input from the entorhinal cortex and send their axon (mossy fibers) to the pyramidal neurons of the CA3 region. The overexpression of TK+ significantly increased the complexity of granule cells compared to control neurons. T1 overexpression by contrast resulted in a slight non significant decrease in dendritic complexity of the granule cells (Figure 15C).

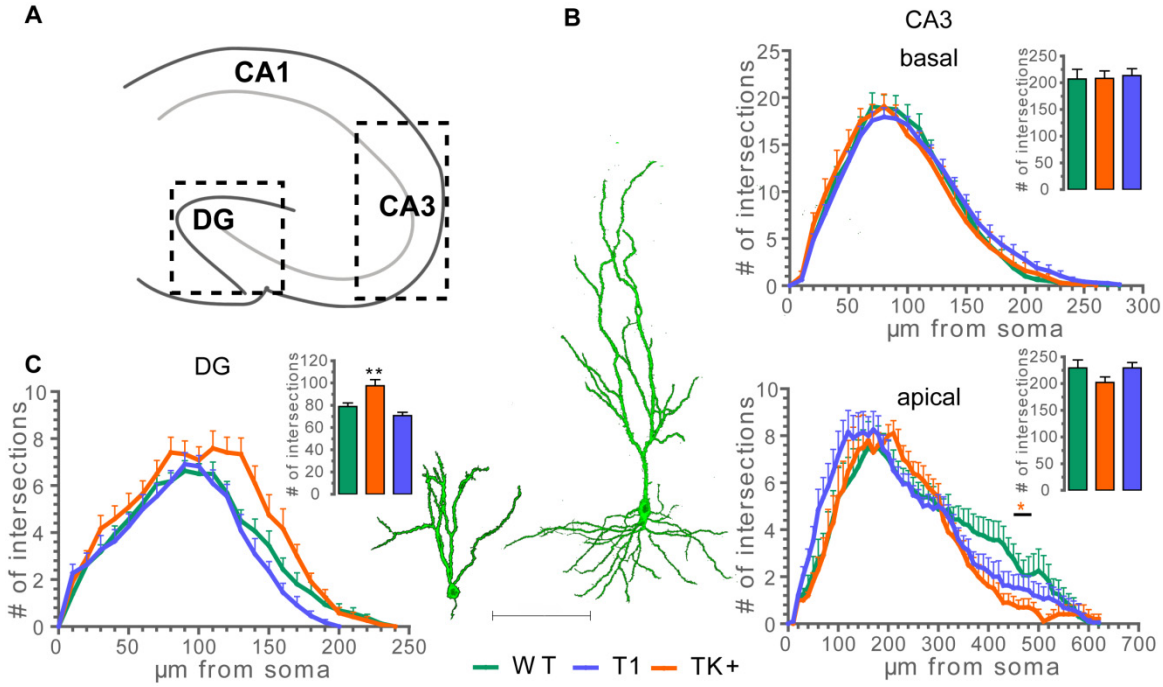


Figure 15 | The overexpression of TrkB receptor splice variants alters neuronal morphology of CA3 pyramidal neurons and dentate granule cells.
A, schematic overview of the hippocampus; **B**, Sholl analysis (basal and apical dendrites) of CA3 pyramidal neurons overexpressing either TrkB.TK+ or TrkB.T1, column charts show the total number of intersections for the different compartments, a CA3 pyramidal neuron can be seen at the left (captured from a maximum intensity projection); the overexpression of TK+ significantly reduces dendritic complexity of the apical dendrite. **C**, Sholl analysis of granule cells in the dentate gyrus, column chart shows the total number of intersections; a granule cell can be seen at the right (captured from a maximum intensity projection); the overexpression of TK+ significantly increases dendritic complexity of granule cells. CA, cornu ammonis, DG, dentate gyrus; scale bar 100 µm; **p < 0.005.

Taken together these observations show that changes in the expression level of different neurotrophin receptors modulate both, dendritic morphology as well as spine number of mature CA1 neurons. In this context, the $p75^{NTR}$ acts as a negative regulator of neuronal morphology. In contrast to this, TK+ positively affects dendrite architecture. The truncated receptor T1 in turn can exert positive as well as negative morphological alterations with respect to dendrites and spines. Notably, the effects of the overexpression of neurotrophin receptors can vary between neuronal subtypes. Specifically, TK+

overexpression in CA3 neurons has the opposite effect than in CA1 or granule cells, namely a reduction in dendritic complexity.

4.1.3 The coexpression of Trkb.T1 and p75^{NTR} compensates the morphological changes elicited by the expression of either one of them

In development, the overall levels of neurotrophins and their receptors determine the balance between survival and apoptosis of neurons (Chao, 2003). The observations made so far in this work indicate that mature neurons, too, depend on precisely regulated levels of neurotrophin receptors. Changes in the expression level of one receptor type induced alteration in neuronal morphology on the level of dendrites and spines. The already proposed roles of p75^{NTR} and TrkB.TK+ as negative and positive modulators of neuronal morphology, respectively, could be further underlined also for mature neurons. Interestingly, the truncated isoform TrkB.T1 seems to play an additional role in this context, mediating both positive and negative structural changes.

To further investigate the interplay of the different types of neurotrophin receptors, coexpression studies in CA1 pyramidal neurons as well as in primary hippocampal cultures were used. Remarkably, an additional overexpression of the p75^{NTR} could be accomplished in slice cultures of transgenic T1 mice, but not in those of transgenic TK+ animals. In contrast to this, viable neurons could be observed in cultures of both transgenic mice after the induction of LTD – a process where it has been suggested that p75^{NTR} activation is involved (Rosch et al., 2005;Woo et al., 2005).

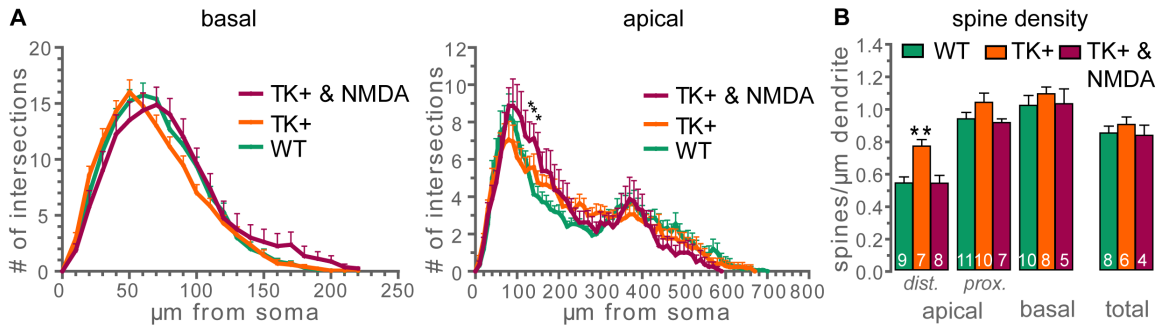


Figure 16 | In neurons overexpressing full-length TrkB the chemical induction of long-term depression significantly increases dendritic morphology but leaves spine numbers unaffected.
A, Sholl analysis (basal and apical dendrites) of transgenic CA1 pyramidal neurons overexpressing full-length TrkB (TK+) (n=8) fixed 48 h after the induction of chemical long-term depression (LTD) via a 10 min application of 20 µM NMDA in comparison to control cells; the induction of LTD leads to a significant increase in dendritic complexity of CA1 neurons overexpressing TK+ compared to WT cells. **B**, spine density of TK+ overexpressing cells after the induction of LTD; spine numbers are not affected by the induction of LTD compared to control cells; *p < 0.05, **p < 0.005.

In CA1 pyramidal neurons overexpressing TK+ the induction of LTD significantly increased dendritic complexity of the apical dendrites (Figure 16A apical) compared to WT neurons. Remarkably, a similar but milder and non significant increase in dendritic complexity could already be observed in untreated TK+ neurons (Figure 12A). Spine numbers in neurons overexpressing TK+ and treated with NMDA were similar to WT cells (Figure 16B, supplement Table S1), indicating that the induction of LTD reversed the TK+-dependent increase in spine density of the distal apical dendrites but left spine density of more proximal compartments unaltered.

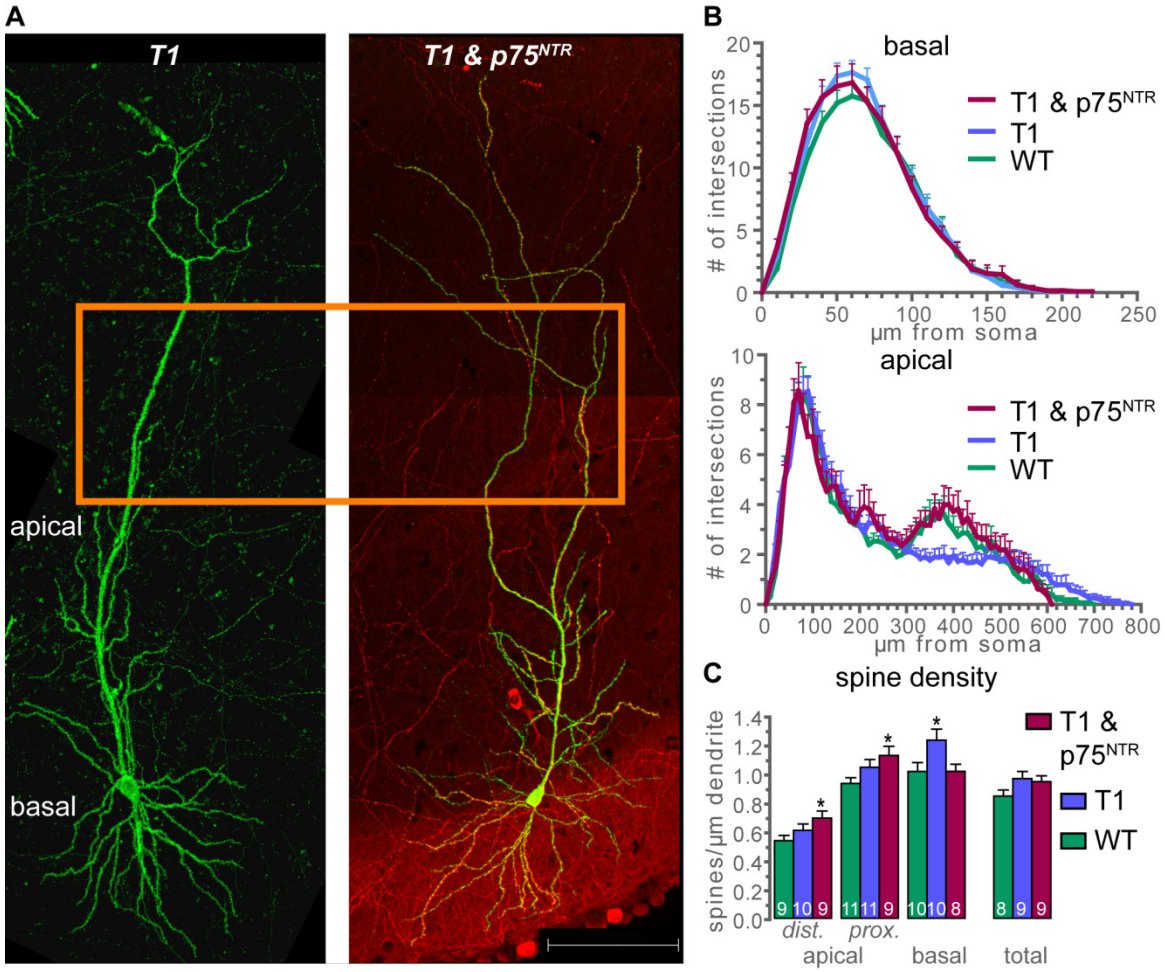


Figure 17 | The additional expression of p75^{NTR} in CA1 neurons overexpressing TrkB.T1 compensates morphological alterations mediated by both receptor types.
A, Maximum intensity projections of a CA1 pyramidal neuron overexpressing transgenic TrkB.T1 (T1) (organotypic cultures, DIV 17) and a cell overexpressing T1 and p75^{NTR} (anti-p75^{NTR} labeled in red), both neurons were transfected with fGFP to allow detailed morphological analysis; the overexpression of both receptors compensates the T1-mediated reduction in dendritic complexity in the mid-apical dendrite (orange box); scale bar 100 µm. **B**, Sholl analysis (basal and apical dendrites) of transgenic CA1 neurons overexpressing T1 or transgenic T1 together with p75^{NTR}; **C**, spine density of control cells, neurons overexpressing T1 and cell overexpressing both T1 and p75^{NTR}, note, that the significant changes in spine density due to the overexpression of T1 are reversed by the concomitant overexpression of p75^{NTR}; *p < 0.05.

Neurons coexpressing both, transgenic T1 and $p75^{NTR}$ showed no signs of degeneration as swellings or retraction bulbs (Figure 17A). Moreover, T1-dependent negative changes in dendritic complexity in the mid-apical dendrites could be restored by the additional expression of $p75^{NTR}$ (Figure 17A orange Box). Indeed, a detailed Sholl analysis revealed that dendritic complexity of neurons overexpressing both neurotrophin receptors was not any longer significantly different from WT cells (Figure 17B apical).

The analysis of spine numbers of CA1 neurons coexpressing T1 and $p75^{NTR}$ revealed a significant increase in spine density of the apical compartment when compared to WT cells (Figure 17C, supplement Table S1). However, a significant increase in spine number of the basal dendrites due to the overexpression of T1 (Figure 13D) could not any longer be detected in neurons overexpressing T1 and $p75^{NTR}$ (Figure 17C).

Interestingly, the chemical induction of LTD in organotypic hippocampal cultures of transgenic T1 mice had a very similar effect than the overexpression of $p75^{NTR}$ in these cultures (Figure 18). Specifically, the Sholl analysis of T1 overexpressing neurons treated with 20 µM NMDA showed no difference compared to WT cells (Figure 18A). This observation indicates that the T1-dependent negative changes in dendritic morphology could be completely restored by the induction of LTD. Spine density as well was found not to be significantly different from the one of WT neurons (Figure 18B, supplement Table S1).

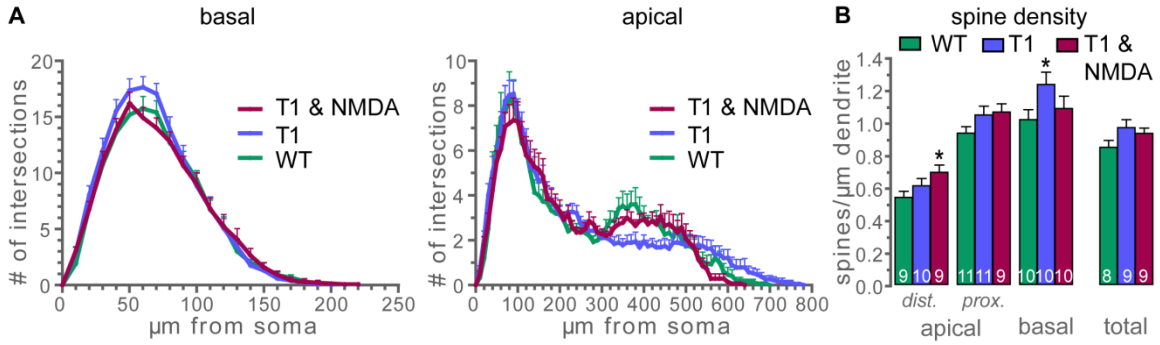


Figure 18 | The induction of long-term-depression can compensate TrkB.T1 induced morphological alterations.

A, Sholl analysis (basal and apical dendrites) and **B**, spine density of CA1 pyramidal neurons of transgenic mice overexpressing TrkB.T1 (T1) as well as neurons overexpressing TrkB.T1 48 h after the chemical induction of long-term depression (LTD) via a 10 min application of 20 µM NMDA; the induction of LTD reverses morphological changes due to the transgenic expression of T1 on the level of both, dendrites as well as on the level of spines; *$p < 0.05$.

In summary, these observation show that the concomitant overexpression of T1 and $p75^{NTR}$ prevents morphological changes elicited by the overexpression of only one of these two receptors. Interestingly, the induction of LTD in T1 overexpressing neurons – and therefore the activation of endogenous $p75^{NTR}$ – had a similar effect.

4.1.4 The extracellular domain of Trkb.T1 is responsible for the compensational effect on $p75^{NTR}$ mediated structural changes

Trk receptors and $p75^{NTR}$ are coexpressed in many neuronal populations. In addition to their often opposing functions in neuronal survival and apoptosis, $p75^{NTR}$ has been shown to enhance ligand sensitivity and specificity of Trk receptors (reviewed in Huang and Reichardt, 2001). If this is attributable to a physical interaction of both receptor types or rather a communication of downstream signaling pathways is still a matter of particular interest (Bothwell, 1995;Bibel et al., 1999;Wehrman et al., 2007).

To further characterize the mutual compensation of T1 and $p75^{NTR}$ on morphological changes elicited by either one of the two receptors, T1 deletions mutants were used (Haapasalo et al., 1999). In this set of experiments, dissociated primary hippocampal neurons were used because of higher transfection efficiency.

In a first step, the overexpression of T1 and $p75^{NTR}$ in primary hippocampal neurons confirmed the results obtained in organotypic slice cultures. Indeed, the overexpression of T1 as well as of $p75^{NTR}$ significantly reduced dendritic complexity of primary hippocampal neurons indicated by a reduction in the number of dendritic endings compared to control cells (Figure 19B, 71 ± 3 dendrites in the control group; 55 ± 4 dendrites in $p75^{NTR}$ overexpressing cells, p=0.0003; 58 ± 3, p=0.004). Moreover, dendritic complexity of neurons overexpressing both T1 and $p75^{NTR}$ was indistinguishable from control cells (Figure 19B, 63 ± 5 dendrites). A similar effect could be observed after the activation of endogenous $p75^{NTR}$ via the chemical induction of LTD in primary hippocampal neurons overexpressing T1. Remarkably, dendritic complexity in these cells was not significantly different from control cells (Figure 19C, 61 ± 5 dendrites in neurons treated with NMDA, 70 ± 4 dendrites in T1 overexpressing cell treated with NMDA).

The expressing of T1 lacking either the extra- or the intracellular domain resulted in a significant simplification of the dendritic tree (Figure 19D, 57 ± 4 dendrites in neurons overexpressing T1-IC, p=0.008; 58 ± 3 dendrites in cells overexpressing T1-EC,

p=0.006). This is especially interesting as the intracellular domain of T1 comprises only 21 amino acids. However, a coexpression of T1 deletions mutants and p75^{NTR} revealed that the mutual compensational effect of both receptors observed before (Figure 19B) is depending on the extracellular domain of T1.

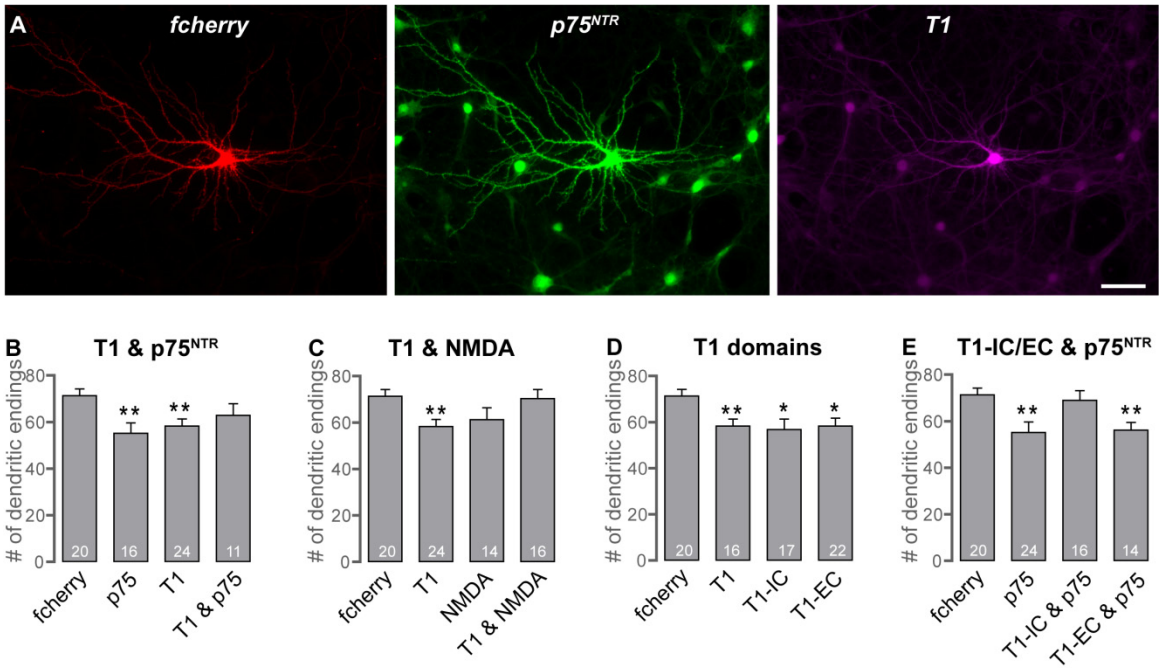


Figure 19 | The compensational effects of a coexpression of TrkB.T1 and p75^{NTR} on neuronal morphology are depending on the extracellular domain of TrkB.T1.
A, Fluorescence images of a primary hippocampal neuron transfected with fcherry, p75^{NTR} and TrkB.T1, scale bar 50 µm; **B**, histogram showing the number of dendritic endings of primary hippocampal neurons transfected with fcherry, p75^{NTR}, T1 or p75^{NTR} and T1; in primary hippocampal cultures the overexpression of T1 or p75^{NTR} significantly reduces dendritic complexity, coexpression of both neurotrophin receptors prevents this reduction. **C**, histogram showing the number of dendritic endings of neurons transfected with fcherry, T1 or treated with 20 µM NMDA for 10 min and fixed 48 h later; as in organotypic cultures, the chemical induction of LTD prevents the T1-dependent reduction in dendritic complexity. **D**, histogram showing the number of dendritic endings of neurons transfected with fcherry, T1 or T1 deletion mutants lacking the extracellular (T1-EC) or the intracellular domain (T1-IC); the T1-mediated reduction in dendritic complexity is depending on the extracellular as well as on the intracellular domain of T1. **E**, histogram showing the number of dendritic endings of cells transfected with fcherry, p75^{NTR} or T1 deletion mutants and p75^{NTR}; the extracellular domain of T1 is necessary and sufficient to rescue the p75^{NTR}-dependent reduction in dendritic complexity; $p < 0.05$, $^{**}p < 0.005$.

Specifically, neurons coexpressing T1 lacking the intracellular domain and p75^{NTR} showed a significantly reduced dendritic tree. Yet, the concomitant expression of T1 lacking the extracellular domain and p75^{NTR} induced no morphological alterations (Figure 19E, 56 ± 3 dendrites in neurons overexpressing T1-EC & p75^{NTR}, p=0.001; 69 ± 4 dendrites in cells overexpressing T1-IC & p75^{NTR}).

In summary, experiments in primary hippocampal neurons confirmed a mutual compensation of T1 and p75^{NTR} with regard to morphological alterations induced by both

neurotrophin receptors. Furthermore it could be shown that this effect is depending on the extracellular domain of T1.

4.2 ProfilinIIa modulates neuronal morphology downstream of $p75^{NTR}$

Structural changes induced by neurotrophin receptors described above are depending on a tightly regulated cytoskeleton. The regulation of microfilament growth, organization or collapse provides neurons with the ability to maintain their structure as well as to allow rapid changes of their morphology.

Profilins are actin-binding proteins known to participate in neuronal actin dynamics, however their precise role in regulating actin-dependent neuronal architecture is still unresolved. In mammalian brains, two isoforms (PFNI and PFNIIa) are expressed. While PFNI is ubiquitous and essential for cellular survival, controversial views exist on a neuronal-specific function of PFNIIa.

4.2.1 RNAi-mediated knockdown of profilinIIa

To investigate the specific function of profilinIIa (PFNIIa) in dendrite morphology and spine stability in mature hippocampal neurons a vector-based RNA interference approach (RNAi) was used. Polycistronic RNAi vectors were designed by Kai Murk (Murk K, 2008) targeting PFNIIa mRNA and expressing fGFP (Figure 20A).

In a second approach, the fGFP-reporter was exchanged for cDNAs encoding for RNAi-resistant PFNIIa, profilinI (PFNI) or PFNIIa mutants lacking specific biochemical properties under a partially deleted CMV promoter (Figure 20A). This deletion was necessary to obtain moderate expression levels as cytotoxicity was shown in HeLa cells upon high levels of PFNIIa expression. Thus, it was possible to simultaneously deplete endogenous PFNIIa and express exogenous profilin-variants. Cotransfection experiments with the PFNIIa-specific RNAi-vector shPFNIIa and PFNIIa expression constructs in HeLa-cells (Murk K, 2008) demonstrated a significant reduction of PFNIIa-level leaving the expression of PFNI unaffected (Figure 20D). In addition, the knockdown of endogenous PFNIIa was confirmed by immunocytochemistry using anti-PFNIIa antibody (Murk K et al., 2009) on primary hippocampal neurons (Figure 20C). Quantification comparing mean pixel intensities of the neuronal soma of shPFNIIa transfected cells and neighbour neurons showed a reduction in PFNIIa protein level of 73.3 ± 2% (Figure 20B).

FGFP or a shRNA expression vector against luciferase were used as negative controls. As no significant differences between both control experiments were observed (supplement Figure S1), the results were combined as control.

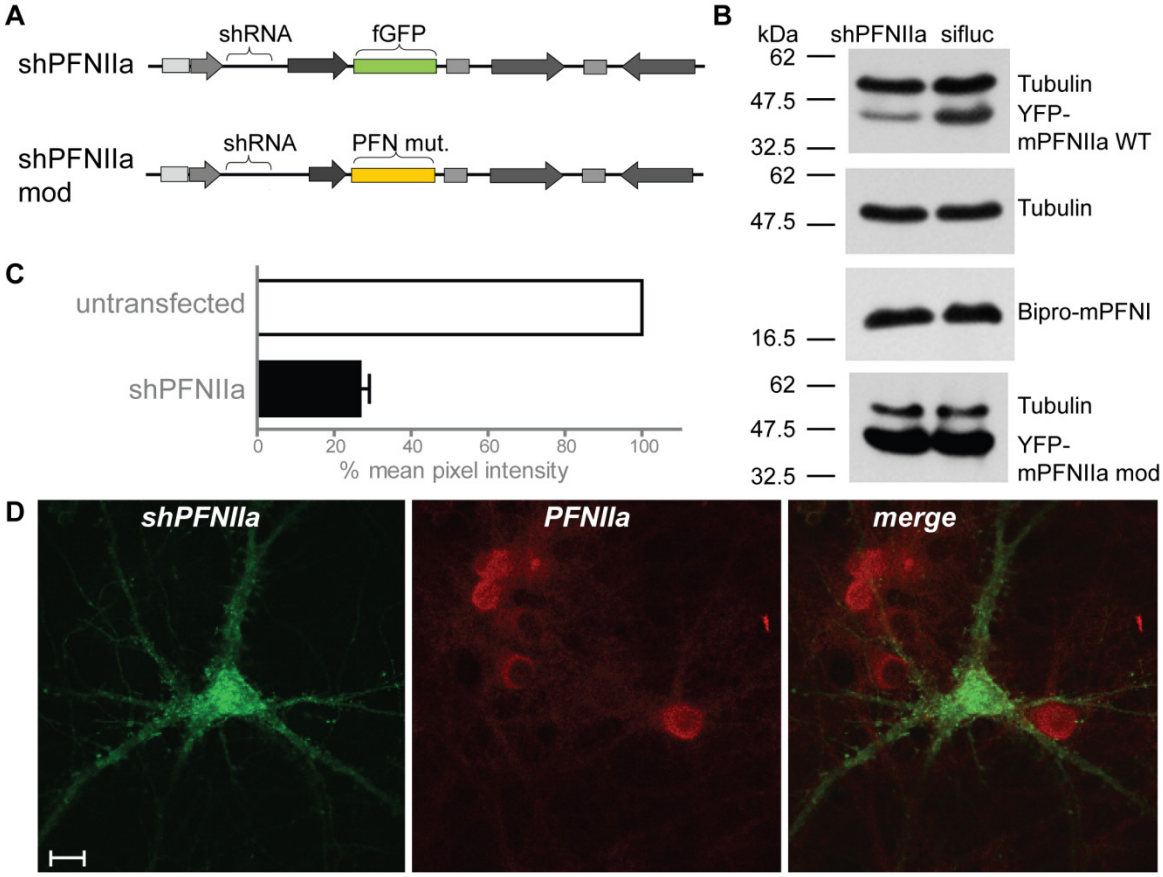


Figure 20 | Knockdown of the brain specific profilinIIa via RNAi
A, polycistronic vector constructs targeting profilinIIa (PFNIIa) mRNA and expressing farnesylated GFP (fGFP) or a modified form of profilinIIa resistant to shRNA binding (YFP-mod) both under a truncated CMV promoter. **B**, specific knockdown of exogenous profilinIIa in HeLa cells, Bipro-tagged profilinI levels are unaffected by the knockdown, control plasmid sifluc does not alter profilinIIa expression levels, modified profilinIIa is resistant to the knockdown; **C**, mean pixel intensity (neuronal soma) of primary hippocampal neurons (14 DIV) transfected with shPFNIIa (n=18) or untransfected neighbouring cells (n=18) stained with anti-PFNIIa, pixel intensity of control cells was set to 100%, PFNIIa protein levels are reduced to 26.7 ± 2%. **D**, fluorescence images of primary hippocampal neurons (14 DIV) transfected with shPFNIIa (green) and labeled with anti-PFNIIa (red); scale bar 10 µm.

4.2.2 The knockdown of profilinIIa reduces dendritic complexity and spine density in CA1 pyramidal neurons

Organotypic hippocampal cultures were transfected using particle-mediated gene transfer at 7 days *in vitro* and fixed 7 days post transfection. CA1 neurons with reduced PFNIIa levels showed no signs of degeneration as swellings or retraction bulbs (Figure 21A).

However, shPFNIIa transfected neurons showed a significantly reduced overall dendritic complexity compared to cells transfected with control plasmids (Figure 21A).

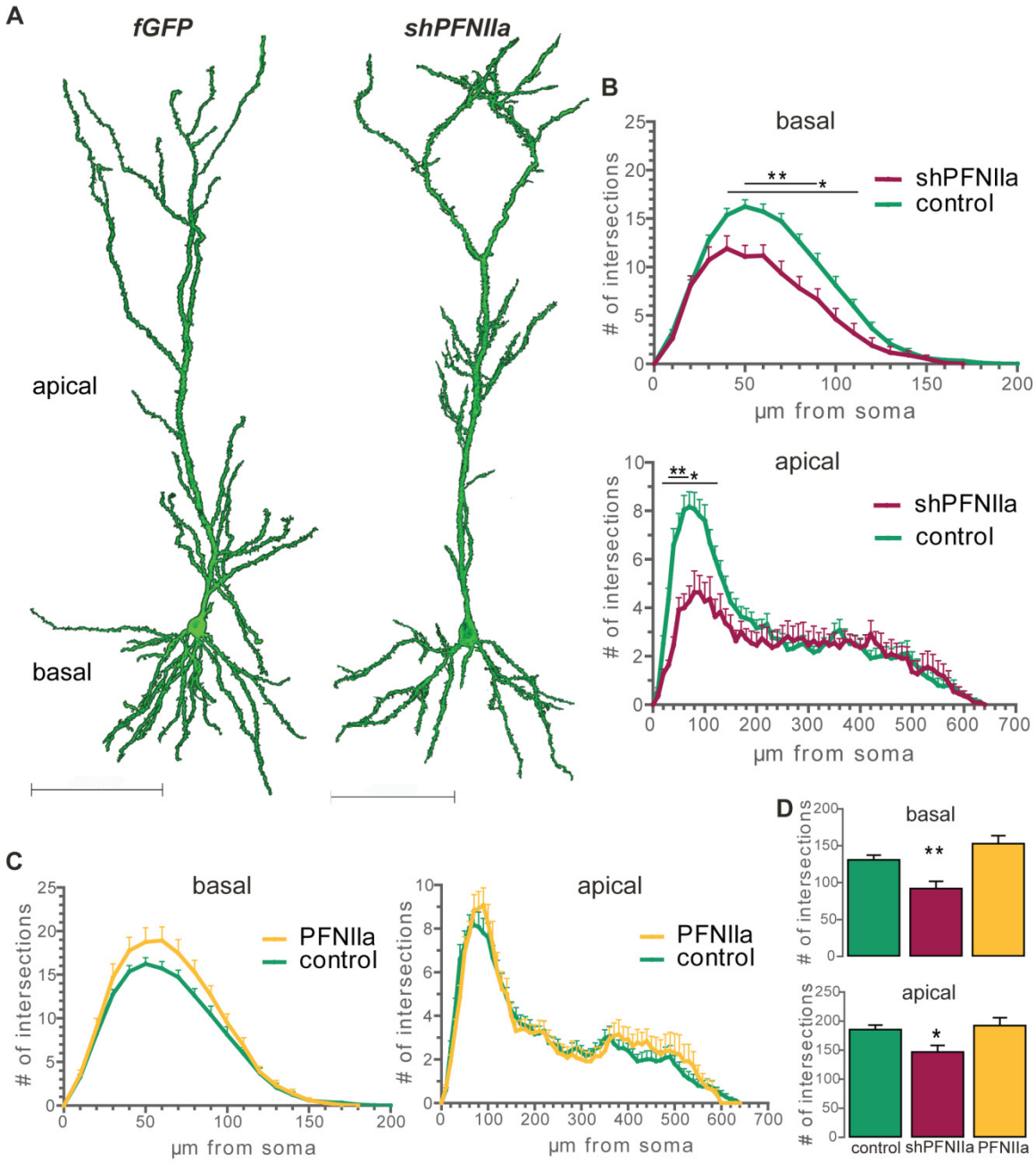


Figure 21 | Knockdown of PFNIIa negatively influences dendritic morphology of CA1 pyramidal neurons.
A, CA1 neurons (14 DIV) transfected with fGFP or shPFNIIa, images were captured from maximum intensity projections, the cells were fixed 7 DPT; scale bar 100 µm; **B**, Sholl analysis (basal and apical dendrites) of shPFNIIa transfected cells (n=11) shows a significant reduction of the dendritic complexity compared to control neurons (n=25). **C**, Sholl analysis (basal and apical dendrites) of control cells and neurons transfected with PFNIIa, expression time 48 h (n=13), shows no significant alterations compared to control cells. **D**, total dendritic complexity of the basal dendrites of control neurons (132 ± 6), shPFNIIa (92 ± 9; p=0.002) and PFNIIa (153 ± 9) expressing cells, as well as total dendritic complexity of the apical dendrites of control neurons (188 ± 7), shPFNIIa (147 ± 10; p=0.005) and PFNIIa (192 ± 13) expressing CA1 neurons shows a significant reduction of shPFNIIa transfected neurons for both compartments compared to control cells; *p < 0.05, **p < 0.005.

Both apical and basal dendrites of shPFNIIa transfected CA1 neurons showed a significant reduction of dendritic intersections when compared to control cells. Remarkably, despite the obvious reduction in dendritic complexity of shPFNIIa transfected cells this construct showed a much higher transfection efficiency than all other constructs used in this study. This observation suggests that the negative influence on morphology is not simply due to neurotoxic side effects of the PFNIIa knockdown.

In a second set of experiments, a gain of function approach was used by overexpressing PFNIIa N-terminal fused to YFP under a partially deleted CMV promoter for 48 h. Cells with an increased PFNIIa level showed a slight but non significant increase in the overall dendritic complexity (Figure 21D). A detailed Sholl analysis of PFNIIa overexpressing CA1 neurons, too, revealed a slight but non significant increase in dendritic complexity in the basal dendritic compartment as well as in the apical dendritic compartment (Figure 21C).

In summary, the gain and loss of function experiments indicate that PFNIIa is indeed an important regulator of CA1 pyramidal neuron morphology in the mature hippocampus.

According to previous reports (Ackermann and Matus, 2003), PFNIIa is targeted to dendritic spines in an activity-dependent manner. Therefore, the number of dendritic protrusions was analyzed in the loss and gain of function approach. Detailed spine density counts were performed for the different dendritic compartments - basal dendrites as well as proximal and distal apical dendritic compartments.

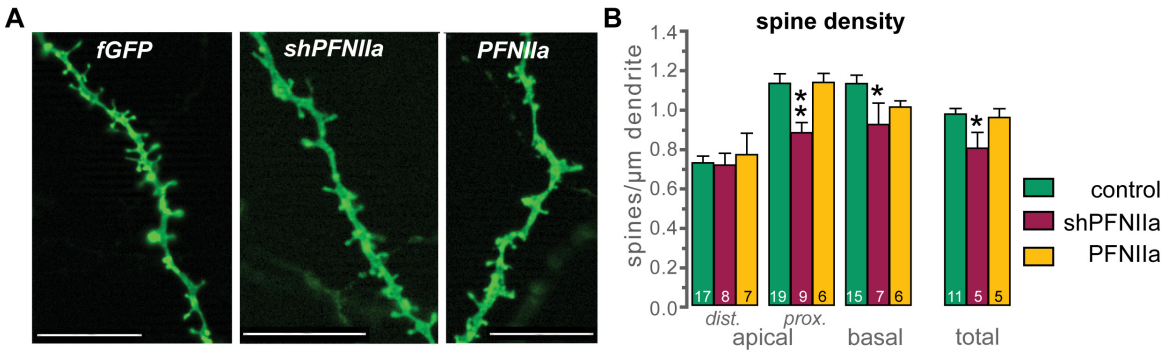


Figure 22 | Spine density is reduced in shPFNIIa transfected CA1 neurons.
A, high resolution images of representative basal dendrites of neurons in organotypic cultures expressing fGFP, shPFNIIa or PFNIIa; **B**, spine density analysis of control cells as well as of cells expressing shPFNIIa or PFNIIa shows a significant reduction in spine number of shPFNIIa transfected neurons; *p < 0.05, **p < 0.005.

As for dendritic complexity, PFNIIa was found to be important for spine maintenance. Specifically, shPFNIIa transfected neurons showed a significantly reduced spine density

of the basal and proximal apical dendrites compared to control neurons (Figure 22A). Total spine density, too, was significantly reduced in shPFNIIa expressing CA1 neurons compared to control cells (Figure 22A, B, supplement Table S2). The overexpression of PFNIIa showed only a mild non significant phenotype in spine density. A slight decrease in spine number could be observed for the basal dendritic compartment while spine density of the apical compartment as well as total spine density were unaltered in comparison to control neurons (Figure 22B).

4.2.3 ProfilinI cannot compensate the reduction in dendritic complexity but in spine density after profilinIIa knockdown

It is still a matter of particular interest why two different profilin isoforms are needed in the central nervous system of vertebrates. In order to investigate if the morphological alterations observed in this study following PFNIIa knockdown in neurons are specific for this isoforms, endogenous PFNIIa was replaced with YFP-PFNI in organotypic hippocampal slice cultures (shPFNIIa & PFNI).

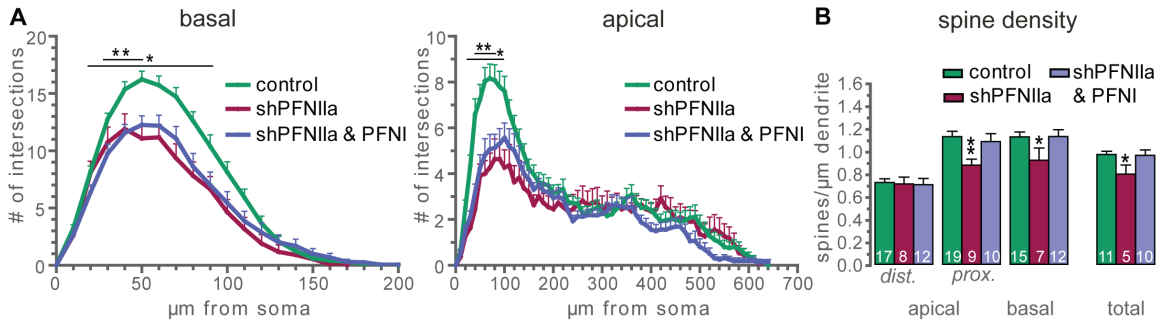


Figure 23 | PFNI cannot rescue shPFNIIa dependent reduction in dendritic complexity but in spine density.
A, Sholl analysis and **B**, spine density of basal and apical dendrites of CA1 pyramidal neurons in organotypic slice cultures expressing shPFNIIa & profilinI (n=16), shPFNIIa or controls plasmids. Remarkably, coexpression of PFNI does not prevent the significant reduction in dendritic complexity but in spine density induced by the knockdown of PFNIIa; *p < 0.05, **p < 0.005; significances shown are always compared to control cells.

CA1 neurons expressing shPFNIIa & PFNI showed an almost identical dendritic phenotype compared to those transfected with shPFNIIa alone. Both in the basal and in the apical compartment dendritic complexity was significantly reduced compared to cells transfected with control plasmids (Figure 23A). However, the loss of spines observed in shPFNIIa transfected cells could be completely prevented by the expression of PFNI.

Spine numbers in neurons expressing shPFNIIa & PFNI were indeed not significantly different from those in control cells (Figure 23B, supplement Table S2).

4.2.4 Actin but not poly-L-proline binding is essential for the profilinIIa-dependent maintenance of dendrites and spines

In a next step, rescue experiments were performed starting with the reintroduction of RNAi-resistant PFNIIa into PFNIIa-deficient neurons. CA1 neurons coexpressing shPFNIIa and RNAi-resistant PFNIIa-mod (shPFNIIa-mod) showed an overall normal dendritic morphology (Figure 24A).

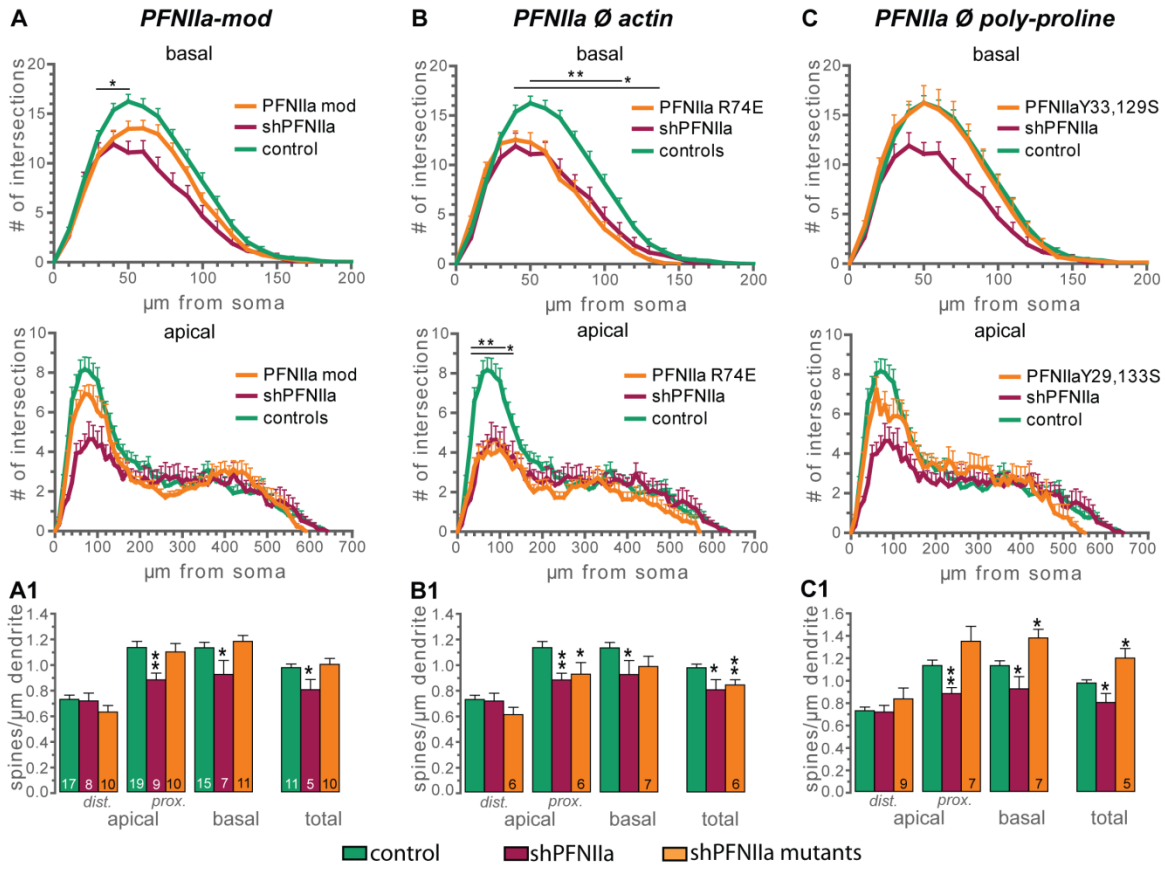


Figure 24 | Reduced dendritic morphology and spine density of shPFNIIa expressing cells can be rescued by expressing RNAi-resistant PFNIIa, the actin-binding site but not the poly-proline-binding site are important for the rescue effect.
A, dendritic morphology and **A1**, spine density of control cells as well as CA1 neurons transfected with shPFNIIa or RNAi-resistant PFNIIa-mod (n=17); the reintroduction of RNAi-resistant PFNIIa-mod. prevents morphological changes induced by the knockdown of PFNIIa. **B**, Sholl analysis of basal and apical dendrites as well as **B1**, spine density of cells expressing controls, shPFNIIa or RNAi-resistant PFNIIa R74E (n=11) which lacks the actin-binding capacity; actin binding is necessary for the resue effect observed in A. **C**, dendritic morphology and **C1**, spine density of control cells and neurons expressing shPFNIIa or RNAi-resistant PFNIIa Y29,133S (n=9) lacking the poly-L-proline-binding capacity; poly-L-proline interaction is not needed for the rescue effect observed in A; *p < 0.05, **p < 0.005, significances shown are always compared to control cells.

Specifically, the Sholl analysis of the apical dendritic compartment was similar to control cells and in the basal dendrites only the region 30-50 µm from soma was significantly reduced compared to control neurons. Moreover, spine density of shPFNIIa-mod transfected cells was almost identical to control spine numbers (Figure 24 A1, supplement Table S2). These results again underline that the effects of PFNIIa knockdown on neuronal morphology are specific for PFNIIa and cannot be seen as unspecific side effects of the RNA interference approach.

To study the role of different PFNIIa binding domains in maintaining dendrite structure and spine stability PFNIIa mutants were used. The first candidate was the actin binding site, where an exchange of an arginin for a glutamate at position 74 led to an impaired actin binding (Murk K, 2008). Endogenous PFNIIa was knocked down using shPFNIIa and at the same the RNAi-resistant PFNIIaR74E (shPFNIIa R74E) was expressed.

The impairment in actin binding led to a highly significant reduction of dendritic complexity in shPFNIIa R74E transfected neurons compared to control cells (Figure 24B). In addition, spine density of shPFNIIa R74E expressing cells was as well significantly reduced in comparison to control neurons (Figure 24B1, supplement Table S2). Both Sholl analysis as well as spine density counts almost completely reproduced the effect observed in neurons depleted of the endogenous PFNIIa.

Other important binding partners for profilin are proteins carrying poly-L-proline (PLP) motives. Therefore a construct was used where tyrosines were exchanged for serines at position 29 and 133 inhibiting poly-proline interaction (shPFNIIa Y29, 133S) (Murk K, 2008). Impairment in PLP-binding did not lead to a reduction of dendritic complexity compared to control cells (Figure 24 C). Interestingly, the expression of PLP-binding deficient PFNIIa led to a significant increase in the number of spines compared to control neurons (Figure 24 C1, supplement Table S2).

4.2.5 ProfilinIIa and profilinI can compensate distinct aspects of p75^{NTR}-dependent morphological alterations

In a next step, it was investigated whether PFNIIa might be involved in the regulation of actin dynamics downstream of known effectors of neuronal morphology as p75^{NTR}. The phenotype of shPFNIIa expressing cells resembled that of neurons overexpressing p75^{NTR} described earlier in this work, showing a comparable reduction of dendritic complexity in the proximal dendritic compartment. In addition, p75^{NTR} has been shown to affect the activity of the small GTPase RhoA (Yamashita et al., 1999;Yamashita and Tohyama, 2003), which in turn regulates dendritic branching as well as spine density (reviewed in Koh, 2007).

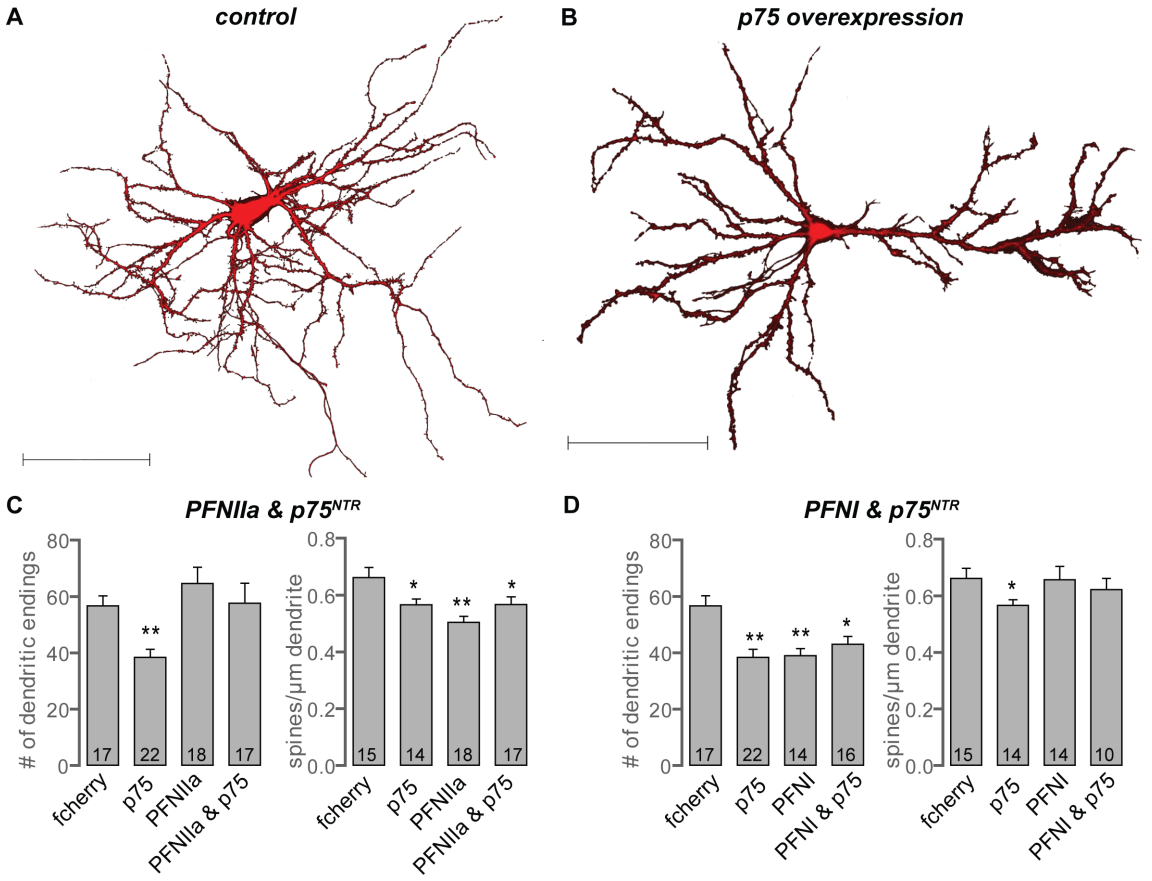


Figure 25 | PFNIIa but not PFNI can compensate p75^{NTR}-dependent dendritic loss in primary hippocampal neurons.
Neurolucida representations of primary hippocampal neurons (21 DIV) expressing farnesylated mcherry (fcherry): **A**, control cell expressing fcherry only and **B**, hippocampal neuron transfected with p75^{NTR} and fcherry; **C**, histograms showing the number of dendritic endings and spine density of neurons expressing only fcherry as a control, p75^{NTR}, PFNIIa or PFNIIa & p75^{NTR}; the expression of PFNIIa prevents p75^{NTR}-dependent loss of dendrites but not of spines; **D**, histograms showing the number of dendritic endings and spine density of cells transfected with fcherry as a control, p75^{NTR}, PFNI or PFNI & p75^{NTR}; the expression of PFNI prevents p75^{NTR}-dependent loss of spines but not of dendrites;*p < 0.05, **p < 0.005; scale bar 100 µm.

Primary hippocampal neurons transfected with $p75^{NTR}$ showed no signs of degeneration as swellings or retraction bulbs compared to control cells (Figure 25 A, B). However, the treatment resulted in a significant simplification of the dendritic tree shown as a reduction in the number of dendrites (Figure 25 C, 56 ± 3 dendrites in the control group versus 38 ± 3 dendrites in $p75^{NTR}$ overexpressing cells, p=0.0003). As the results of the current study indicate that PFNIIa might be important for the maintenance of dendritic complexity, it was investigated whether the expression of PFNIIa would be sufficient to rescue the $p75^{NTR}$-mediated loss of dendrites. Indeed, when $p75^{NTR}$ and PFNIIa were overexpressed within the same cells, the number of dendrites was identical to the one in control neurons, (Figure 25 C, 58 ± 7 dendrites in cells overexpressing $p75^{NTR}$ & PFNIIa). Furthermore, the expression of PFNIIa in dissociated cultures partially reproduced the increase in number of dendrites observed in organotypic cultures, (Figure 25C, 64 ± 6 dendrites). Spine density counts were performed for control cells and neurons overexpressing $p75^{NTR}$, PFNIIa or both proteins. The number of spines on $p75^{NTR}$ overexpressing cells was significantly reduced (Figure 25C, 0.66 ± 0.03 spines/ µm dendrite in control neurons compared to 0.57 ± 0.02 spines/ µm dendrite in $p75^{NTR}$ overexpressing cells, p=0.03). In contrast to the dendritic phenotype, $p75^{NTR}$-dependent spine loss was not rescued by overexpressing PFNIIa within the same neurons (Figure 25C, 0.50 ± 0.03 spines/ µm dendrite in PFNIIa overexpressing neurons, p=0.001; 0.57 ± 0.03 spines/ µm dendrite in $p75^{NTR}$ and PFNIIa overexpressing cells, p=0.04).

Taken together these results show that PFNIIa is able to rescue the reduction in dendritic complexity induced by the overexpression of the $p75^{NTR}$. Remarkably, the $p75^{NTR}$ induced loss of spines was not prevented by the overexpression of PFNIIa.

As a next step, it was investigated whether the inhibition of $p75^{NTR}$-dependent morphological alterations would be PFNIIa specific. Therefore PFNI was expressed alone in dissociated cultures as well as PFNI together with $p75^{NTR}$. The analysis of the total dendritic endings revealed that PFNI could not rescue dendritic simplification induced by $p75^{NTR}$ overexpression (Figure 25D, 43 ± 3 dendrites in cells overexpressing $p75^{NTR}$ and PFNI, p=0.007). Moreover the overexpression of PFNI alone in dissociated cultures significantly reduced the number of dendrites when compared to control neurons (Figure 25D, 39 ± 2 dendrites in cells overexpressing PFNI, p=0.0003). Surprisingly, the number of spines in $p75^{NTR}$ and PFNI coexpressing neurons was observed to be similar to control cells indicating that PFNI prevented the $p75^{NTR}$-dependent loss in the number of dendritic protrusions Figure 25D, 0.62 ± 0.04spines/ µm dendrite in PFNI & $p75^{NTR}$

overexpressing neurons). The expression of PFNI alone did not lead to a significant change in spine number (Figure 25D, 0.66 ± 0.04 spines/ µm dendrite in PFNI overexpressing neurons).

In summary, these observations showed that both profilin isoforms had complementary effects on mature hippocampal neurons. While the overexpression of PFNI reduced the number of dendrites but left spine numbers unaffected, the overexpression of PFNIIa slightly increased dendritic complexity and at the same time led to a loss of dendritic spines. Furthermore, these results suggest a possible and most likely separate role for both profilin isoforms downstream of the p75^{NTR}.

5 DISCUSSION

Over hundred years ago Santiago Ramón y Cajal described for the first time the growing tip of an axon. Since then numerous scientists spent their life working on the molecules and mechanisms that determine neuronal architecture. A striking feature of the cells of our nervous system is the complexity of their cell shape. After the initial formation of synaptic connections the morphology of a neuron does not remain static. Neuronal networks are, in fact, further refined during development by selectively removing projections to incorrect targets. Remarkably, the ability to add or remove synapses, to grow or retract neurites is not lost during the entire lifespan of a neuron. Moreover, the function of the adult brain depends on a tightly regulated balance between stability and plasticity. This ensures reliable signal transduction on the one hand and allows on the other hand changes in the connectivity of neuronal circuits that can be considered as neural representations of memory in the brain.

How could neuronal structure be regulated to fulfill these often opposing needs?

During my work, I concentrated on two different sets of molecules both known to be important for the morphology of neurons: 1) Neurotrophins and their receptors – initially described as survival factors of neurons – were found to be involved in almost all aspects of the function of vertebrate neurons, ranging from differentiation to synaptogenesis and activity-dependent forms of synaptic plasticity. The variety of cellular functions they exert assigns them as key molecules providing neurons with the potential to maintain as well as to change their functional and structural properties. In the first part of this work, I investigated whether the balance in expression levels and activation of different neurotrophin receptors could modulate the morphology of mature pyramidal neurons.

2) In order to affect neuronal cell shape, the receptors located at the plasma membrane need to signal to the underlying cytoskeleton. From the very beginning of neuritogenesis to the subtle modifications in spine shape, a multitude of molecules is involved in regulating microfilament growth, organization and collapse. Among these molecules, profilin plays a key role by binding to G-actin and providing it to the growing actin filament. However, the role of the brain specific form profilinIIa remains so far elusive. In the second part of my work, I asked the question, why two different profilins could be needed in the central nervous system.

5.1 Neurotrophin receptors as modulators of neuronal morphology

The cell-biological function of neurotrophins depends on two distinct types of receptors: the tropomyosin-related kinase receptors (Trk) and the pan neurotrophin receptor $p75^{NTR}$. Whereas the Trk receptors exert well-characterized trophic functions, the versatile actions of $p75^{NTR}$ range from promoting cell survival and differentiation to the induction of apoptosis (reviewed in Reichardt, 2006;Blochl and Blochl, 2007). In contrast to the tyrosine kinase carrying Trk receptors $p75^{NTR}$ is devoid of intrinsic kinase signaling. Interestingly, both TrkB and TrkC exist in different splice variants (Klein et al., 1990;Middlemas et al., 1991;Tsoulfas et al., 1993). While the full-length tyrosine kinase is well characterized, the role of the other truncated kinase-lacking isoforms remains so far elusive.

In the first part of my work, I focused on the role of the three most abundant types of neurotrophin receptors in the murine hippocampus – TrkB full-length (TK+), truncated TrkB.T1 (T1) and $p75^{NTR}$ – in modulating the morphology of pyramidal neurons.

5.1.1 The expression levels of neurotrophin receptors differentially modulate neuronal morphology in mature pyramidal neurons

In order to investigate morphological alterations due to changes in the expression levels of the neurotrophin receptors, organotypic cultures (DIV 17) of transgenic mice overexpressing either full-length or truncated TrkB (Saarelainen et al., 2000b;Koponen et al., 2004a) were used. To avoid developmental side effects, expression of the transgene is driven by the Thy-1.2 promoter and starts around postnatal day ten (Caroni, 1997). Interestingly, the endogenous expression patterns of both TrkB splice variants in the rodent brain differ substantially. TK+ is already expressed at early embryonic stages and the expression of the receptor is reported to increase rapidly to adult levels around birth. In contrast to this, T1 expression is very low during embryonic and postnatal development and reaches adult levels between p5 and p20 (Fryer et al., 1996). Remarkably, the increase in T1 expression coincides with extensive gliogenesis and the stabilization of synaptic circuits. A comparison of the mRNA expression levels of both isoforms reveals a general pattern where TK+ is the predominant form early in development, whereas T1 mRNA predominates at later stages (Fryer et al., 1996). Interestingly, the ratios of the full-length and the truncated isoforms differ between

distinct brain regions, with septum and hippocampus showing the highest ratio of T1 to TK+ (Fryer et al., 1996). It is still a matter of particular interest if this increase in T1 expression levels is mostly attributable to an increased expression in glia cells. Yet an ever increasing number of publications report that T1 is indeed expressed in neurons (Escandon et al., 1994;Allendoerfer et al., 1994;Armanini et al., 1995;Fryer et al., 1996;Kryl et al., 1999;Ohira et al., 1999;Ohira et al., 2004;Silhol et al., 2005;Silhol et al., 2007).

TK+ as a positive modulator of neuronal morphology

In this study, the overexpression of TK+ resulted in a mild and non significant increase in the dendritic complexity of CA1 pyramidal cells. This increase was restricted to the proximal dendritic compartment, a phenotype already described in ferret cortical slices (Yacoubian and Lo, 2000). The authors showed that the overexpression of TK+ leads to a significant increase in dendritic complexity of the proximal dendrites. Moreover, the addition of TrkB ligands BDNF and NT-4/5 further promotes the phenotype, indicating that the amount of available ligand is substantial for the morphological changes observed (Yacoubian and Lo, 2000). Spine density in TK+ overexpressing neurons was significantly increased, however, only at the distal apical dendrites. Taken together these results show that indeed the overexpression of TK+ positively modulates the morphology of mature CA1 pyramidal neurons. In addition, TK+ overexpression increased dendritic complexity of dentate granule cells. These observations are in line with the already proposed role for TK+ as a positive modulator of both functional as well as structural plasticity (McAllister et al., 1995;Kang and Schuman, 1995;Korte et al., 1995b;Shimada et al., 1998;Tyler and Pozzo-Miller, 2003). Moreover transgenic TK+ mice have already been reported to show facilitated learning and an increase in the mRNA levels of plasticity related genes as c-fos and jun-B (Koponen et al., 2004a;Koponen et al., 2004b). However, the lack of a more dramatic phenotype in the current study indicates that the action of transgenic TK+ might at least in part depend on limiting availability of BDNF in the organotypic cultures. In addition, compensatory mechanisms might restrict the excessive activation of TK+ mediated signaling pathways (Yacoubian and Lo, 2000;Klau et al., 2001;Lahteinen et al., 2002). Thus, TK+ overexpressing mice display a reduced expression of the α-CaMKII mRNA, a molecule known to play an important role in plastic changes during LTP (Koponen et al., 2004a). The decrease in dendritic complexity of CA3 pyramidal neurons observed in the current study further promotes the idea that the

overexpression of TK+ could indeed induce compensatory mechanisms, not only on the functional level, but also in neuronal structure.

Ambivalent action of TrkB.T1

In contrast to the phenotype observed in organotypic cultures of transgenic TK+ mice, the transgenic overexpression of TrkB.T1 resulted in a significant decrease of dendritic complexity of CA1 pyramidal neurons. Alterations in dendritic morphology were restricted to the mid-apical compartment. Moreover, spine density of T1 overexpressing neurons was significantly increased in the basal dendrites of CA1 neurons compared to control cells. It is an interesting finding that the truncated form of TrkB – lacking intracellular kinase activity – is able to modulate neuronal architecture. This has already been reported by different studies, yet with distinct effects of T1 expression on neuronal or glia cell morphology (Haapasalo et al., 1999;Yacoubian and Lo, 2000;Hartmann et al., 2004;Ohira et al., 2005;Chakravarthy et al., 2006;Ohira et al., 2007;Carim-Todd et al., 2009). In ferret cortical slices, T1 has been found to inhibit the outgrowth of new branches but to promote the elongation of preexisting ones in developing neurons (Yacoubian and Lo, 2000). This is at least in parts in line with the phenotype of transgenic T1 neurons analyzed in this study, which are more mature. Cells with an increased level of T1 were significantly less complex than control cells, indicating that T1 might inhibit the formation of new branches. At the same time, T1 overexpressing neurons were longer than control cells.

In addition to the alterations in dendritic morphology, spine density of the basal dendrites was significantly increased. This finding is supported by earlier studies showing the induction of filopodia formation due to an increased level of T1 (Haapasalo et al., 1999;Hartmann et al., 2004). However, T1 overexpressing neurons analyzed in the current study showed no changes in spine subtype composition compared to WT cells. This observation indicates that in more mature neurons, T1 overexpression induces the formation of new spines instead of filopodia. Moreover, the increase in spine number of the basal dendrites in T1 overexpressing neurons might be correlated to an increase in synapse number, as the relative number of mushroom spines (which bear a bulbous spine head) was the same as in WT cells. The size of the spine head can in general be correlated to the size of the PSD and the number of postsynaptic receptors (reviewed in Hering and Sheng, 2001). Thus mushroom spines can be seen as the predominant spine type which carries functionally mature synapses. Further colocalization studies using pre- and

postsynaptic marker proteins as synapsin and PSD95 are needed in order to correlate the increase in total spine number to the number of synapses.

Since the discovery of truncated Trk receptors, there is an ongoing debate about their physiological function *in vivo.* Ample evidence indicates that they might act as dominant negative receptors by heterodimerizing with the full-length forms thereby preventing autophosphorylation and downstream signaling (Eide et al., 1996;Drake et al., 1999;Ohira et al., 2001;Haapasalo et al., 2001;Haapasalo et al., 2002;Lahteinen et al., 2002). Indeed, transgenic mice overexpressing T1 show an impairment in long-term spatial memory (Saarelainen et al., 2000b). There are conflicting data about alterations in synaptic plasticity in these mice. Initially, LTP has been shown to be normal in hippocampal slices of T1 overexpressing mice (Saarelainen et al., 2000b), but more recent experiments revealed a significant impairment in long-term potentiation (Buschler, 2007).

This could provide one possible explanation for the T1-induced structural alterations observed in this study. T1 binding to full-length TrkB might result in a decreased activation of TrkB and a subsequent loss of dendrites in hippocampal CA1 neurons. It has been reported that haploinsufficiency of both TrkB and TrkC negatively influences neuronal structure in the hippocampus and amygdala (Bohlen und Halbach O. et al., 2003). The decrease in TrkB signaling could also be attributable to a reduced availability of ligand. Indeed, T1 was shown to act a BDNF scavenging receptor, thereby limiting the amount of available BDNF (Klein et al., 1990;Middlemas et al., 1991;Biffo et al., 1995;Eide et al., 1996;Saarelainen et al., 2000a). Carim-Todd at al. (2009) showed a partial rescue of BDNF-haploinsuffiency by T1 deletion suggesting that indeed T1 may limit BDNF signaling under physiological conditions in vivo. T1 might be part of a mechanism critical in preventing pathological activation of TK+, by functioning as a buffer to prevent overactivation of TK+ following increased neuronal activity. In addition, the authors further report that the lack of a strong developmental phenotype in T1 knockout mice supports the idea that T1 is not involved in promoting neuronal survival (Carim-Todd et al., 2009). Interestingly, T1 deficiency leads to a reduction in neuronal complexity in the amygdala which may be in parts responsible for increased anxiety in T1 knockout mice (Carim-Todd et al., 2009). The finding that both the overexpression as well as the reduction in T1 levels induces negative structural changes in neurons points to the fact that the precise expression level of T1 or the T1 expression level relative to the other types of neurotrophin receptors TK+ and $p75^{NTR}$ might indeed be important for neuronal architecture.

Spine density is increased in T1 overexpressing CA1 neurons, a phenotype which cannot easily be explained by a dominant negative effect of T1 on TK+. Spine density might be increased as a compensational effect for the loss of dendrites. Alternatively, a more exciting explanation might be found in the observation that T1 has been reported to be capable of signaling independently (Baxter et al., 1997;Rose et al., 2003;Ohira et al., 2005;Cheng et al., 2007). A direct signaling role for T1 is unexpected as its short intracellular domain comprises only 21 amino acids and is devoid of intrinsic catalytic activity. Yet, earlier studies using microphysiometric essays indicated that both truncated forms T1 and T2 are capable of ligand-mediated changes in cell physiology and that the isoform-specific intracellular sequences are necessary for this response (Baxter et al., 1997). T1 activation by BDNF has been shown to stimulate Ca^{2+} signaling in astrocytes most likely mediated by an unknown G-protein (Rose et al., 2003). Moreover, other proteins were identified to bind to the cytoplasmatic tail of T1, especially a so far unknown protein named TTIP (truncated TrkB interacting protein) (Kryl and Barker, 2000). Interestingly, Rho-GDI1, too, has been found to be an interaction partner of T1, thereby providing a possible link to the actin cytoskeleton for T1 to induce changes in astrocyte morphology (Ohira et al., 2005;Ohira et al., 2007). Taken together these results show that indeed an alternative explanation for the morphological alterations observed in this study might reside in T1 signaling, probably by affecting the actin cytoskeleton in a Rho-GTPase-dependent manner.

$P75^{NTR}$ as a negative modulator of neuronal morphology

The overexpression of $p75^{NTR}$ in the current study significantly reduced dendritic complexity as well as spine density in CA1 pyramidal neurons, a phenotype already described before for hippocampal neurons (Zagrebelsky et al., 2005). In a loss of function approach, the gene targeting of $p75^{NTR}$ has been shown to lead to an increase in spine density and dendritic complexity (Zagrebelsky et al., 2005).

The growth inhibiting function of $p75^{NTR}$ is at least in part mediated by the small GTPase RhoA. Specifically, unoccupied $p75^{NTR}$ is reported to activate RhoA, thereby negatively regulating neurite outgrowth (Gehler et al., 2004). Neurotrophin binding to $p75^{NTR}$ in turn ceases RhoA activity and therefore stimulates axonal growth (Yamashita et al., 1999).

It is important to mention that the negative morphological alterations mediated by $p75^{NTR}$ affected different dendritic compartments on CA1 pyramidal neurons. Whereas spine density was reduced in the basal compartment, dendritic complexity was decreased in the apical dendrites. In this respect the overexpression of T1 had a similar effect inducing

different morphological alterations in the basal and apical dendrites (increase in spine density of the basal dendrites, decrease in complexity of the apical dendritic compartment). While differences between CA1 and CA3 pyramidal neurons in the hippocampus are well-characterized (Spruston, 2008), not much is known about the differences in the dendritic trees on a single pyramidal cell (basal versus apical dendrites). Notably, it was published recently that indeed functional compartments can be discriminated on CA1 pyramidal neurons (Sajikumar et al., 2007). Protein synthesis following the induction of LTP was found to be restricted to basal or apical dendrites for different molecules. These observations indicate that in fact the protein contents of basal and apical dendrites can be different and might therefore be responsible for the different neurotrophin receptor mediated effects observed in the current study.

In summary, I could show that changes in the expression level of single neurotrophin receptor types in the murine hippocampus significantly interfere with the overall normal morphology of mature CA1 pyramidal neurons, both on the level of dendrites as well as of spines. The results for TrkB.TK+ and $p75^{NTR}$ are in line with their already described roles as respectively positive and negative regulators of neuronal morphology. TrkB.T1, however, can be seen as an ambivalent receptor mediating both positive and negative morphological alterations. If this is due to a dominant negative effect on TK+ or independent signaling of the receptor still needs to be further investigated.
These findings suggest that mature neurons use a tightly balanced expression of neurotrophin receptors to control their morphology. To further investigate the interplay of the different types of neurotrophin receptors, coexpression studies in CA1 pyramidal neurons were used.

5.1.2 Mutual inhibition of TrkB.T1 and $p75^{NTR}$

The interaction of neurotrophins with Trk receptors has generally been considered to be of high-affinity. However, it is now clear that the binding of NGF to TrkA and of BDNF to TrkB is of low-affinity, but can be modulated by receptor dimerization, structural modifications or association with the $p75^{NTR}$ (reviewed in Chao, 2003). The molecular nature of this so called high-affinity binding sites between Trk receptors and $p75^{NTR}$ and moreover the interplay of the different types of neurotrophin receptors still remains enigmatic. Several studies presented functional and structural evidence for the formation

of high-affinity binding sites leading to an increased ligand sensitivity and specificity. Specifically, a direct physical interaction of Trk receptors and p75 was suggested in many studies (Hempstead et al., 1991;Mahadeo et al., 1994;Lee et al., 1994;Horton et al., 1997). Interestingly, although some groups were able to coimmunoprecipitate this dual receptor complex (Huber and Chao, 1995;Bibel et al., 1999) others failed to do so (Jing et al., 1992).

To analyze the effect of a potential interplay between $p75^{NTR}$ and either truncated or full-length TrkB on neuronal morphology, two different approaches were used: 1) the concomitant expression by biolistical transfection of transgenic slice cultures of TK+ or T1 with $p75^{NTR}$, and 2) the activation of $p75^{NTR}$ via the chemical induction of NMDA receptor dependent long-term depression (LTD). LTD in transgenic slice cultures was induced by applying 20 µM NMDA for 10 min (Woo et al., 2005). LTD maintenance is impaired in $p75^{NTR}$ knockout mice, indicating its crucial role in this form of negative synaptic plasticity (Rosch et al., 2005;Woo et al., 2005).

TrkB.TK+ and p75^{NTR}

Whereas CA1 neurons expressing both T1 and $p75^{NTR}$ showed no signs of degeneration, a coexpression of $p75^{NTR}$ with the full-length TrkB receptor could not be accomplished. This finding indicates that indeed the expression of both receptors might lead to the formation of high-affinity receptors eventually resulting in the overstimulation of TK+ and a subsequent induction of apoptosis. The use of apoptosis detection methods (tunnel essay or immunohistochemistry against active caspases) in slice cultures transfected with both neurotrophin receptors could help to further clarify this hypothesis.

Interestingly, the induction of LTD by NMDA application in transgenic TK+ cultures resulted in a significant increase in dendritic complexity compared to control cells. In addition, the loss of spines in the proximal dendrites of control cells following LTD induction could not be observed in TK+ overexpressing CA1 neurons. The activation of endogenous $p75^{NTR}$ in these experiments using this stimulation protocol (Woo et al., 2005) seemed to enhance the TK+-dependent increase in dendritic complexity to a significant value. A TK+-induced increase in spine density, however, could not be observed any longer after the induction of LTD. Taken together these results in part underline again a cooperation of TK+ and $p75^{NTR}$ in positively modulating neuronal morphology. Yet, this effect could not be shown on the level of spines.

TrkB.T1 and p75^{NTR}

The coexpression of T1 and $p75^{NTR}$ had a completely different effect than the one described above for the full-length TrkB receptor. Remarkably, whereas the overexpression of one of the two receptors led to significant alterations in neuronal morphology, dendritic complexity of CA1 pyramidal neurons overexpressing both receptors was not significantly different from control cells. Moreover, significant changes in spine number due to the overexpression of one receptor type were absent in neurons overexpressing both receptors. The induction of LTD in transgenic T1 slice cultures had a similar effect than the overexpression of $p75^{NTR}$. Specifically, the T1 induced changes in dendritic complexity and spine density were completely rescued by $p75^{NTR}$ overexpression. These results pointed to a mutual inhibition of the two receptors, opening the speculation of whether this could be due to a physical protein-protein interaction or to partly converging signaling pathways. A model of the putative cooperation of T1 and $p75^{NTR}$ can be seen in Figure 26.

The expression of T1 deletion mutants in primary hippocampal neurons revealed that the intracellular domain of T1 is not essential for the rescuing effect of T1 on $p75^{NTR}$-mediated structural changes. This result does not support the hypothesis that the observed mutual inhibition of both receptors is due to an overlap in signaling pathways for example on the level of activation of different members of the Rho-GTPase family. Interestingly, Rho-GDI1 was found to associate with both receptors (Yamashita and Tohyama, 2003;Ohira et al., 2005). However, further experiments have to clarify if at least some of the T1-induced morphological changes observed in this study could be mediated by T1 signaling. For example blocking the interaction of T1 with its intracellular binding partners as Rho-GDI1 could help to answer this question.

$P75^{NTR}$ cooperates with a variety of cell-surface receptors, such as the Nogo receptor, sortilin or the Trk receptors to activate diverse intracellular signaling cascades (reviewed by Barker, 2004). It was reported recently, that NGF binding to $p75^{NTR}$ leads to conformational changes in the NGF dimer which makes the association of a second $p75^{NTR}$ molecule in a 1:2:1 complex impossible as it has been reported for the Trk receptors (He and Garcia, 2004). The observation of this asymmetric NGF/ $p75^{NTR}$ complex further promotes the idea that $p75^{NTR}$ and Trk receptors could bind simultaneously to one neurotrophin dimer. The relative expression levels of both types of neurotrophin receptors would therefore result in the formation of homo- or heterodimers activating distinct intracellular pathways. However, recent evidence indicates that the binding footprint of TrkA and $p75^{NTR}$ on NGF overlap in a way that prevents them from

binding simultaneously (Wehrman et al., 2007). Data from an enzyme complementation essay to detect reversible protein-protein interactions in intact cells used in this study show homodimerization of TrkA or $p75^{NTR}$ but failed to detect heterodimers of the two receptors (Wehrman et al., 2007). Indeed, coimmunoprecipitation results for Trk receptors and $p75^{NTR}$ in most cases used antibodies against the intracellular domain of Trk receptors and could therefore be misleading due to contamination by intact endosoms carrying Trk receptors.

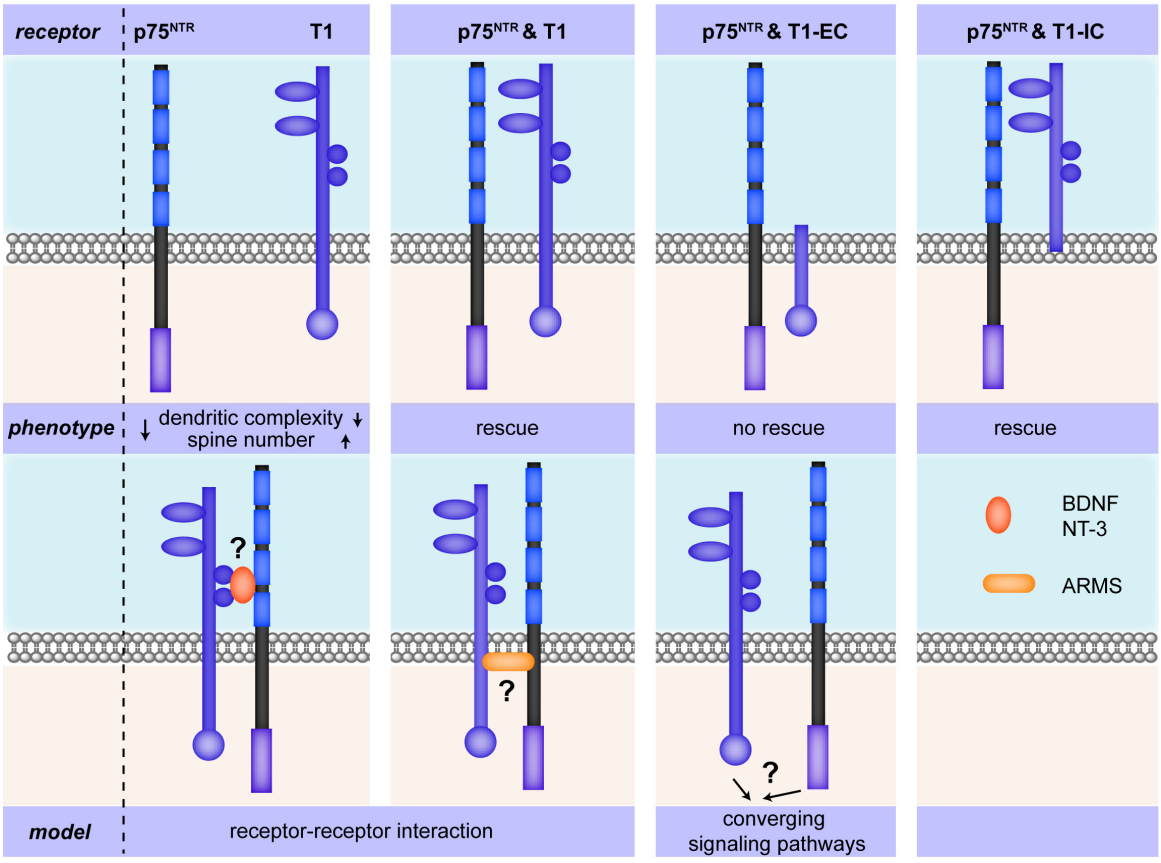


Figure 26 | Models for the interaction of TrkB.T1 and $p75^{NTR}$
The expression of $p75^{NTR}$ and T1 in organotypic hippocampal slice cultures and primary hippocampal neurons significantly affected neuronal morphology on the level of dendrites and spines (upper panel). However, a concomitant expression of both receptors reversed these morphological changes. This effect is depending on the extracellular domain of T1 but not the intracellular part (upper panel). The lower panel shows putative models how both neurotrophin receptors could interact to compensate each other's action.
ARMS, ankyrin-rich membrane spanning protein; BDNF, brain derived neurotrophic factor; ↓decrease; ↑increase NT, neurotrophin; TrkB.T1-EC, T1 lacking the extracellular domain; TrkB.T1-IC, T1 lacking the intracellular domain

What kind of interaction occurring between these two distinct receptors could account for their complex crosstalk still needs to be clarified. However, different models exist such as receptor clustering in lipid raft, potential ligand-passing mechanisms or the interaction via different intracellular adaptor molecules (Wehrman et al., 2007;Barker, 2007).

The results of the current study shed new light on the function of the truncated TrkB receptor T1. Evidence can be provided for a role of T1 not only as the dominant negative form of the TrkB full-length receptor but as well as a receptor that can compensate $p75^{NTR}$-mediated structural changes. T1 expression and transport to the membrane could be used to tightly regulate the action of neurotrophin receptors especially as T1 is predominantly expressed in the adult brain, where changes in neuronal structure should be restricted to regions of neuronal plasticity. While heterodimers of T1 and TK+ have been already reported (Ohira et al., 2001), the nature of the interaction of $p75^{NTR}$ and T1 still remains elusive. As a physical receptor interaction carrying the neurotrophin ligand is questionable, T1 and $p75^{NTR}$ might interact – and block each other's action – directly. A different explanation could be found in the existence of adaptor molecules like the ankyrin-rich membrane spanning protein (ARMS), that has been found to cluster neurotrophin receptors (Chang et al., 2004). Further experiments are needed to reveal the mechanism of interaction between T1 and $p75^{NTR}$.

5.2 ProfilinIIa and profilinI cooperate in regulating distinct aspects of dendrite structure downstream of $p75^{NTR}$

5.2.1 Why do neurons need two profilins?

The existence of two different isoforms of profilin in the mammalian brain long since raised the question why two forms could be necessary, especially as only PFNI has been shown to be essential for cell survival (Witke et al., 2001). Although PFNIIa is expressed predominantly in the brain, PFNI shows high expression levels there, too, indicating that both proteins might have crucial and distinct actions (Witke et al., 1998). Interestingly, the ratios of PFNIIa and PFNI vary significantly between different brain regions, however, nowhere in the adult central nervous system either of them is expressed alone (Lambrechts et al., 2000). The ratio of PFNIIa to PFNI is especially high in hippocampus and cortex. (Lambrechts et al., 2000).

Recent studies in PFNIIa knockout mice reported an overall normal brain anatomy and neuronal morphology. Processes of functional plasticity, as long-term potentiation (LTP) and long-term depression (LTD) as well as learning are normal in these mice. These findings led the authors to suggest a predominantly presynaptic role for PFNIIa in controlling vesicle exocytosis (Pilo-Boyl P. et al., 2007). However, compensatory effects cannot be ruled out, as may be suggested from the observation of an initial, but transient increase in the number of sprouting neurites from young PFNIIa knockout neurons (Da Silva et al., 2003).

By using RNAi-mediated acute knockdown of PFNIIa in mature pyramidal neurons, the postsynaptic role of PFNIIa could be analyzed without possible compensatory effects. Both the number of dendrites as well as the number of spines was reduced in PFNIIa deficient pyramidal neurons suggesting it to play a crucial role in actin-dependent stability of these structures. This is consistent with studies in non-neuronal cells where profilins have been shown to regulate the stability of the actin cytoskeleton by increasing the density of submembranous actin networks thereby stabilizing these dynamic actin structures (Finkel et al., 1994;Rothkegel et al., 1996).

Changes in dendritic complexity and spine density were observed primarily in the proximal parts of the dendritic tree – basal dendrites as well as the proximal apical dendrite. In this respect it is interesting to note that proximal dendrites of CA1 neurons

receive input from a different area than distal dendrites (Figure 27). Specifically, whereas the proximal dendrites are innervated by the Schaffer collaterals from the CA3 region, the distal apical tufts of CA1 neurons receive input via the perforant path from the entorhinal cortex (Figure 27). The connection to the entorhinal cortex is disrupted in organotypic hippocampal slice cultures whereas the CA3 region is still intact. Spontaneous activity within the slice cultures indeed might be restricted primarily to the local circuit from the CA3 to the CA1 region. The stabilizing function of PFNIIa in mature CA1 neurons of organotypic hippocampal cultures could therefore be especially relevant in the proximal dendritic compartment receiving the main input from the CA3 region versus the apical tufts receiving input mainly from the entorhinal cortex. Neuronal plasticity provides nerve cells with the ability to react to certain stimuli with changes on the level of synaptic efficacy, eventually leading to structural modifications (reviewed by Yuste and Bonhoeffer, 2001). Such structural plasticity processes have been shown to depend on the variable amount of F-actin within dendritic spines (Fukazawa et al., 2003;Chen et al., 2007) (for reviews see Matus, 2000;Lamprecht and LeDoux, 2004). Moreover, the use of FRAP and photoactivatable GFP indicates a rapid actin turnover in dendritic spines that is regulated by activity (Star et al., 2002;Honkura et al., 2008). Thus, depletion of PFNIIa and its actin filament assembly promoting activity might affect predominantly the proximal dendritic compartment of CA1 pyramidal neurons.

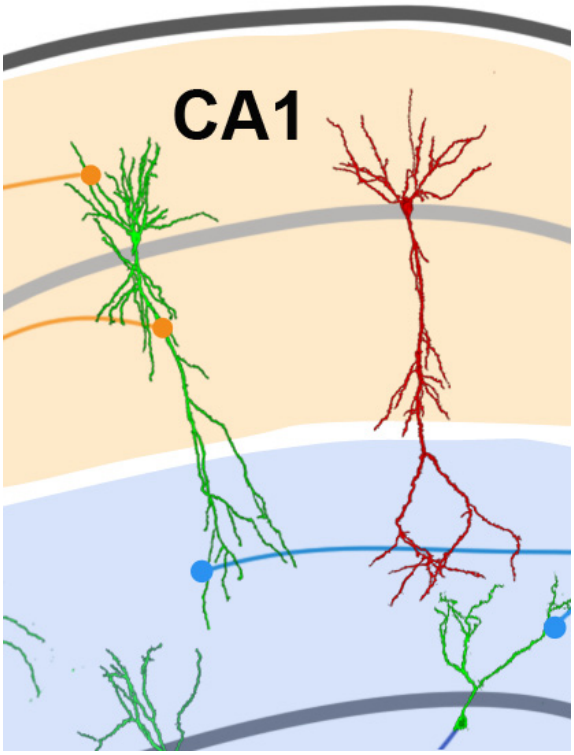


Figure 27 | Schematic illustration showing the domains of synaptic input in the hippocampal CA1 area
Depicted are two CA1 pyramidal neurons: a control (green) and a PFNIIa-deficient cell (red). Basal dendrites as well as proximal apical dendrites receive input from the CA3 region via the Schaffer collaterals (orange), whereas the apical tufts receive input from the entorhinal cortex via the perforant path (blue). Note that the reduction in dendritic complexity of the PFNIIa-deficient neuron in restricted to the proximal parts. An overview about the whole hippocampal circuit can be seen in Figure 2.

A crucial question addressed in the current study is whether the morphological changes observed before following PFNIIa knockdown are isoform specific. Therefore an RNAi-vector replacing endogenous PFNIIa with exogenous YFP-PFNI was transfected into

hippocampal CA1 neurons. Remarkably, the decrease in PFNIIa-specific dendritic morphology could not be prevented by the replacement with exogenous PFNI. However, the spine number in shPFNIIa & PFNI expressing neurons was comparable to control levels. Using this gene replacement approach it was possible to discriminate between a specific function of PFNIIa in stabilizing dendrite architecture and a redundant action of the two profilin isoforms in maintaining dendritic spines.

As a next step, the discrimination of PFNI and PFNIIa should be further defined under conditions that were known to involve changes in neuronal morphology. $P75^{NTR}$ was chosen as a candidate molecule known to be a negative modulator of neuronal morphology (see above) and moreover known to signal via RhoA and ROCK to the cytoskeleton (Yamashita et al., 1999;Yamashita and Tohyama, 2003).

5.2.2 ProfilinI and profilinIIa are part of a signaling cascade downstream of $p75^{NTR}$

The overexpression of $p75^{NTR}$ in primary hippocampal neurons resulted in a highly significant reduction in the number of dendrites and a significant loss in dendritic spines. Subsequent rescue experiments using either PFNI or PFNIIa revealed differences in the action of both isoforms: PFNIIa prevented the reduction in dendritic morphology but not in spine number whereas expression of PFNI had an opposite effect, rescuing the loss in spine density but not in dendritic complexity. Hence, it could be clearly discriminated between the cellular function of the two profilin isoforms downstream of $p75^{NTR}$: PFNIIa mediating dendrite architecture while PFNI maintains spine stability downstream of $p75^{NTR}$. At the same time these experiments propose a first hint about how $p75^{NTR}$ could specifically mediate changes in different aspects of neuronal morphology by signaling to different actin binding molecules. $P75^{NTR}$ itself is a negative regulator of axonal elongation and neurite outgrowth by constitutively activating RhoA, while neurotrophin binding to $p75^{NTR}$ counteracts this effect by inhibiting RhoA activity (Yamashita et al., 1999;Yamashita and Tohyama, 2003;Gehler et al., 2004). The current results support this model, as neurons overexpressing $p75^{NTR}$ show a reduction both in dendritic complexity and spine number, notably reproducing the phenotype observed in PFNIIa deficient neurons. $P75^{NTR}$ overexpression could inhibit PFNIIa- or PFNI-mediated actin polymerization and therefore induce the loss of dendrites and spines. In this model, the overexpression of $p75^{NTR}$ would activate RhoA followed by the activation of the

downstream Rho-kinase ROCK. This is in agreement with the fact, that the phosphorylation of profilin by ROCK has been shown to decrease profilin-actin interaction (Shao et al., 2008). Presumably, this reduces actin polymerization and could therefore be responsible for the observed retraction of dendrites. Remarkably, the $p75^{NTR}$ dependent loss in spine number could not be prevented by PFNIIa but by PFNI instead. One explanation for this finding might reside in the differential distribution of the two isoforms between different cellular compartments. Although both proteins have been shown to accumulate in spine heads following depolarization (Ackermann and Matus, 2003;Neuhoff et al., 2005), this shift has been shown only for endogenous PFNI, while Ackermann and Matus used an overexpression approach. To answer these questions the expression patterns of profilins need to be further defined using high resolution imaging techniques as immunogold labeling in EM studies. Another possible explanation for the differences in the rescue experiments using PFNI or PFNIIa is based on the fact that while both isoforms share several binding partners, there are also isoforms-specific ligand proteins for both profilins, like Wave1, dynamin or ROCK (Witke et al., 1998;Witke, 2004).

5.2.3 PLP-containing ligands of PFNIIa are involved in the regulation of spine numbers in pyramidal neurons

In order to distinguish between the actin-binding capacity of profilin and the interaction with poly-L-proline (PLP) containing ligands, it could be shown that PLP binding does not seem to be necessary for the PFNIIa-dependent maintenance of dendrites and spines. Moreover, impaired PLP-binding of PFNIIa significantly increased spine numbers. At first sight this seemed contradictory as PLP-containing profilin ligands are known to mediate profilin-actin interaction with the growing filament and are therefore important for actin polymerization (Holt and Koffer, 2001). As the PLP-binding mutant was able to rescue the shPFNIIa phenotype and even more to significantly increase spine numbers above the control level, PLP-profilin ligands and profilins might tightly regulate each other in controlling actin polymerization. Upon disruption of this interaction, profilin-actin as well as PLP ligands are free to independently promote actin polymerization leading to enhanced actin filament growth. This view is supported by experiments in PC-12 cells concerning PFNI, where cells expressing PLP-binding deficient PFNI mutants displayed more and longer neurites (Lambrechts et al., 2006). One example of a PLP-

ligand in neurons might be the interaction of PFNIIa with ROCK leading to the possible inactivation of PFNIIa due to phosphorylation (see above). In the PLP-binding mutant the interaction and subsequent inactivation of PFNIIa by ROCK would be prevented and would therefore lead to enhanced actin filament polymerization.

Taken together, these results shed new light on the importance of specific profilin isoforms in controlling the morphology of mature neurons in the central nervous system of mammals. Using gene replacement of PFNIIa a specific function of PFNIIa in the maintenance of dendritic structure in mature hippocampal neurons could be revealed. In addition, the results further promote the idea that profilins and at least some of their PLP-containing ligands can tightly regulate each other and thereby control actin dynamics within neurons. Finally, both PFNI (for spines) and PFNIIa (for dendrites) could be identified as signaling molecules downstream $p75^{NTR}$ mediating its actions on dendrites versus spines independently.

5.3 Conclusions and outlook

In the current study, I was able to show that neurotrophin receptors can significantly interfere with the morphology of mature pyramidal neurons of the mouse hippocampus. In line with the Yin-Yang model proposed by Lu (2005), I could provide evidence that neurons can use the ratio of TrkB (Yang) to $p75^{NTR}$ (Yin) to induce the growth or retraction of dendrites and spines. This dual receptor system might be regulated even more precisely by the expression of the truncated receptor TrkB.T1. Expression and insertion of T1 into the plasma membrane could tightly restrict the action of both types of neurotrophin receptors: TrkB and $p75^{NTR}$. The results of the current study indicate that indeed T1 could act as a dominant negative inhibitor of the $p75^{NTR}$ action. However, the nature of an interaction between both receptors still needs to be clarified. Coimmunoprecipitation of T1 together with $p75^{NTR}$ or the identification of molecules that might act as linkers between both receptors will help in future experiments to reveal details about their interaction. In addition, loss-of-function experiments in T1 and $p75^{NTR}$ knockout mice could be used to obtain further insights into how the balance of neurotrophin receptors affects neuronal morphology.

In my work, I already started to draw a connection between structural changes in neuronal architecture mediated by neurotrophin receptors and actin dynamics in these cells modulating their morphology. First of all, I could show that the actin-binding protein PFNIIa is essential for the maintenance of dendrite structure in mature pyramidal neurons. Moreover, I was able to discriminate between a unique function of the brain-specific isoform PFNIIa for the structure of dendrites and a redundant function of both PFNI and PFNIIa in spine maintenance. Most notably, both profilin isoforms seem to be involved in the regulation of actin dynamics downstream of $p75^{NTR}$ mediating it's action independently on dendrites and spines. To reveal details about how $p75^{NTR}$ could signal to profilins several experiments are needed in the future. One possible signaling mechanism could be the activation of RhoA and its downstream kinase ROCK, which in turn could lead to the phosphorylation of profilin, thereby inhibiting actin polymerization. Blocking of RhoA and ROCK activity in $p75^{NTR}$ overexpressing neurons will give a first hint, if these molecules are indeed involved in mature pyramidal neurons to mediate $p75^{NTR}$-dependent structural changes. In addition, coimmunoprecipitation experiments of ROCK together with profilin could reveal a possible interaction. Analysis of whether profilins

are primarily phosphorylated or dephosphorylated depending on the expression level of $p75^{NTR}$ (in p75NTR knockout mice or neurons overexpressing the receptor) could provide insight into regulatory mechanisms of the putative inhibition of profilin-dependent actin polymerization by $p75^{NTR}$.

A different set of experiments can help to better define the action of $p75^{NTR}$ and profilins under more physiological conditions. The induction of LTD via the application of 20 µM NMDA in organotypic hippocampal slice cultures in the current study was used to activate the endogenous $p75^{NTR}$. As this treatment results in the loss of spines, it would be interesting to investigate if this phenotype will be prevented by the overexpression of PFNI or PFNIIa.

Live imaging experiments using different stimulus protocols (to induce LTP or LTD) in neurons either overexpressing PFNIIa or depleted of this protein can provide further insight to what extend PFNIIa might be involved in activity-dependent structural plasticity acting as a mediator between the action of neurotrophin receptors and the neuronal cytoskeleton.

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7 SUPPLEMENT

7.1 Supplementary data

7.1.1 Detailed spine numbers

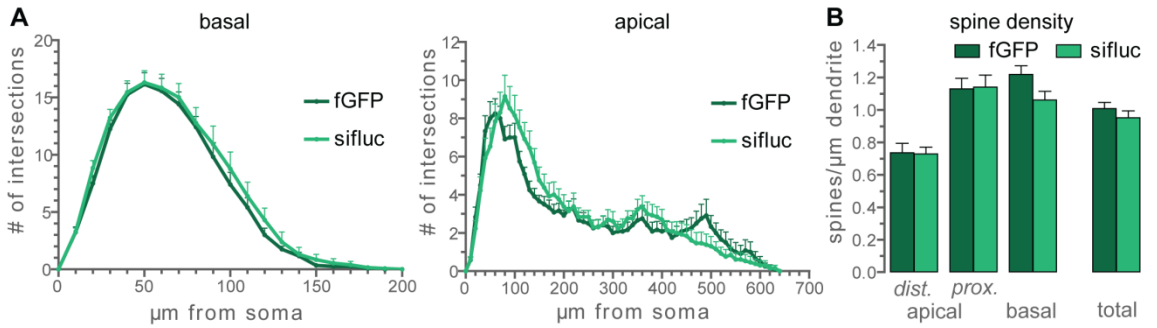
Table S1 | Spine numbers of organotypic cultures for Results 3.1.
Spine numbers are assigned as spines per µm dendrite, p-values indicated in brackets are always compared to control experiments

Experiment (Fig.)	dist. apical	prox. apical	basal	total
WT (9)	0.55 ± 0.03	0.94 ± 0.04	1.02 ± 0.06	0.85 ± 0.04
WT & p75 (9)	0.57 ± 0.04	0.93 ± 0.05	0.82 ± 0.02 *(0.02)*	0.78 ± 0.03
WT & NMDA (10)	0.58 ± 0.04	0.78 ± 0.05 *(0.02.)*	0.84 ± 0.05 *(0.04)*	0.74 ± 0.04
trg. T1 (12)	0.61 ± 0.04	1.04 ± 0.06	1.23 ± 0.07 *(0.04.)*	0.98 ± 0.05
trg. TK+ (11)	0.77 ± 0.05 (0.003)	1.04 ± 0.05 *(n.s.)*	1.10 ± 0.03 *(ns)*	0.91 ± 0.04
trg. TK+&NMDA (15)	0.54 ± 0.05	1.04 ± 0.07 *(n.s.)*	0.92 ± 0.02 *(n.s.)*	0.84 ± 0.03
trg. T1 & p75 (16)	0.70 ± 0.05	1.13 ± 0.06 *(n.s.)*	1.03 ± 0.04 *(n.s.)*	0.95 ± 0.04
trg. T1 & NMDA (17)	0.70 ± 0.04	1.07 ± 0.05 *(n.s.)*	1.09 ± 0.07 *(n.s.)*	0.94 ± 0.03

Table S2 | Spine numbers of organotypic cultures for Results 3.2.
Spine numbers are assigned as spines per µm dendrite, p-values indicated in brackets are always compared to control experiments

Experiment (Fig.)	dist. apical	prox. apical	basal	total
fGFP & sifluc (21)	0.73 ± 0.03	1.14 ± 0.05	1.14 ± 0.04	1.02 ± 0.02
shPFNIIa (21)	0.72 ± 0.06	0.88 ± 0.05 *(0.004)*	0.93 ± 0.10 *(0.04)*	0.82 ± 0.07 *(0.006)*
shPFNIIa & PFNI (22)	0.71 ± 0.05	1.09 ± 0.06 *(n.s.)*	1.13 ± 0.06 *(n.s.)*	0.97 ± 0.04 *(n.s.)*
shPFNIIa mod. (23)	0.63 ± 0.05	1.10 ± 0.06 *(n.s.)*	1.18 ± 0.04 *(n.s.)*	1.00 ± 0.04 *(n.s.)*
shPFNIIa R74E (23)	0.61 ± 0.05	0.93 ± 0.08 *(0.04)*	0.99 ± 0.07 *(0.08)*	0.84 ± 0.03 *(0.003)*
shPFNIIa Y29,133S (23)	0.83 ± 0.09	1.35 ± 0.12 *(n.s.)*	1.38 ± 0.07 *(0.007)*	1.20 ± 0.07 *(0.02)*

7.1.2 shRNA luciferase control



Supplementary Figure S1 | Control experiments for the shPFNIIa expression in neurons.
Sholl analysis (basal and apical dendrites) of CA1 neurons in organotypic hippocampal slice cultures (17 DIV) transfected with a control vector expressing shluciferase (sifluc) or fGFP as control experiments **B**, spine density of CA1 neurons expressing sifluc or fGFP.

7.2 Table of figures

7.3 **Abbreviations**

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	brain derived neurotrophic factor
CA1/CA3	cornu ammonis (hippocampal subfields)
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CMV promoter	human cytomegalovirus immediate-early promoter
DG	dentate gyrus (hippocampal subfield)
GABA	γ-aminobutyric acid
GBSS	Gey's balanced salt solution
LTD	long-term depression
LTP	long-term potentiation
NGF	nerve growth factor
NMDA	N-methy-D-aspartic acid
NT	neurotrophin
$p75^{NTR}$	pan neurotrophin receptor p75
PFNI	profilinI
PFNIIa	profilinIIa
PLP	poly-L-proline
RNAi	RNA interference
ROCK	Rho kinase
Rho GDI1	Rho GDP dissociation inhibitor 1
shRNA	short hairpin RNA
T1-EC	TrkB.T1 lacking the extracellular domain
T1-IC	TrkB.T1 lacking the intracellular domain
Trk	tropomyosin related kinase receptor
TrkB.T1 (T1)	truncated TrkB receptor T1
TrkB.T2	truncated TrkB receptor T2
TrkB.TK+ (TK+)	full-length TrkB receptor
WT	Wild type

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